Is melanoma associated leucoderma (MAL) a distinct entity compared to classial vitiligo?

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Abstract

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Keywords: H_2O_2, $ONOO^-$, p53, p21, p76, MDM4, MDM4phospho, SPARC, TGF- β1, VEGF-A

Patients with classical vitiligo lose partially their protecting inherited pigment. The cause of the disease is still unknown. Despite massive epidermal oxidative / nitrative stress and signs for DNA-damage in the skin and in the plasma, these patients have no higher prevalence for sun induced non-melanoma skin cancer and increased photo-damage. Protection and DNA-repair have been attributed to a functioning up-regulated wild type p53 / p21 cascade in association with up-regulated p76 ^{MDM2}. As some patients with cutaneous melanoma develop depigmentations away from their primary tumour site post surgical excision, it became of our interest, whether this melanoma associated leucoderma (MAL) is the same as classical vitiligo. The purpose of this thesis was two-fold. In part I, we wanted to further substantiate the reasons behind the constantly up-regulated wild-type functioning p53 / p21 cascade in classical vitiligo utilising a panel of proteins with direct and / or indirect action on p53 regulation, including p21, $p76^{MDM2}$, MDM4/MDM4phospho, SPARC, VEGF-A and TGF-β1. In part II, we wanted to characterize MAL and compare this peculiar leucoderma with classical vitiligo using the same protein panel and methodologies. To achieve our goals, we used *in vivo* FT-Raman spectroscopy, *in vitro* cell cultures, *in vitro* and *in situ* immuno-fluorescence labelling, Western blot, dot blot and computer modelling techniques. Our data showed distinct differences between classical vitiligo and MAL.

Our results in MAL exhibited a concentration dependent protein expression gradient between the basal / suprabasl layers and the upper layers of the epidermal compartment using catalase, $ONOO^-$, p53, p21, MDM4, $p76^{MDM2}$, TGF-β1 and VEGF-A expression gradient. Moreover, we document for the first time the presence of a nitrated non-fuctional SPARC protein in classical vitiligo which is absent in MAL. Although we show *in vivo* considerable ROS / RNS- mediated stress in MAL and classical vitiligo documented by FT-Raman spectroscopy, Western blot and *in situ* immuno-fluorescence, our results prove that MAL and classical vitiligo are two distinct entities.

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All work presented in this thesis is dedicated to the spirit of my best friend and my best tutor, my late father, Chemist, Mr Ahmed Tharwat Elsayed. I believe that he would be proud of what I have achieved. I am very proud being his daughter until the end of my life.

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$^1O_2$	Singlet oxygen
5-cysdopa	5-cysteine-S-YL-dopa
$6BH_4$	(6R)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin
8-oxo-dG	8-oxo- 7, 8-dihydro-2‘-deoxyguanine
8-oxo-G	8-oxo- 7, 8-dihydro-2‘- guanine
AC	actinic keratoses
AchE	acetylcholinesterase
ACTH	adrenocorticotropic hormone
AP1	adaptor protein1
AP3	adaptor protein 3
APE1	apurinic / apyrimidinic endonuclease 1
Arf	alternate reading frame
Asp	asparagine
ATM	ataxia telangiectasia mutated
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
ATR	ataxia telangiectasia and Rad3-related protein
BAX	Bcl-2–associated X protein
BCC	basal cell carcinoma
BchE	butyrylcholinesterase
Bcl-2	B-cell lymphoma 2
BER	base-excision repair
bFGF	basic fibroblast growth factor
BM-40	basement-membrane-40
BMZ	basement membrane zone
BSA	bovine serum albumin
CAT	catalase

CDC25A	cell division cycle 25 homologue A
CDK	cyclin-dependent kinase
CHK1,2	check point kinases 1 and 2
CMV	cytomegalovirus
CIs	confidence intervals
DAPI	4, 6-diamino-2-phenylindole
DHI	5, 6, dihydroxyindole
DHICA	5, 6-dihydroxyindole-2carboxylic acid
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithioerythriol
EBV	Epstein Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ERK	extracellular regulated kinase
FITC	fluorescein isothiocyanate
FS	follistatin
GADD45	growth arrest DNA damage inducible 45
GPx	glutathione peroxidase
GR	glutathione reductase
GV	generalized vitiligo
GWAS	Genomewide association studies
H_2O_2	hydrogen peroxide
HATs	histone acetyltransferases
HCV	hepatitis C virus
HDAC2	histone deacetylase 2
hOgg1	8-oxoguanine-DNA glycosylase 1
HR	hazard ratio
ILVs	intralumenal vesicles

kDa	kilo Dalton
L-DOPA	3, 4-dihydroxy-L- phenylalanine
LTBP	latent TGF-beta binding protein
MAL	melanoma associated leucoderma
MC1-R	melanocortin-1 receptor
MC4-R	melanocortin-4 receptor
MDM2/4	mouse double minute
MITF	microphtalmia-associated transcription factor
TPH	tryptophan hydroxylase
MLph	melanophilin
mRNA	messenger ribonucleic acid
MSR	methionine sulfoxide reductase
MT	microtubule
MYO5A	myosin 5A
NADPH	nicotinamide adenine dinucleotide phosphate
NES	nuclear export signal
NDS	normal donkey serum
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localisation signals
NMSCs	non-melanoma skin cancers
NO	nitric oxide
NoLS	nucleolar localization signal
NSV	non –segmental vitiligo
$O_2^{\cdot -}$	superoxide radical
OA1	oculocutaneous albinism type 1
ORs	odds ratios
OCT	optimal cutting temperature compound
$^{\cdot}OH$	hydroxyl radical
$ONOO^-$	peroxinitrite
P/S	penicillin/streptomycin

p15	cyclin-dependent kinase 4 inhibitor B (CDKN2B)
p21	cyclin-dependent kinase inhibitor
p53C	p53central DNA-binding domain
PACE4	paired basic amino-acid-cleaving enzyme-4
PAH	phenylalanine hydroxylase
PBS	phosphate buffered saline
PC1	prohormone convertase 1
PC2	prohormone convertase 2
PC-KUS	pseudocatalase Karin U Schallreuter
PDGF	platelet derived growth factor
PNMT	phenylethanolamine N-methyltransferase
POMC	pro-opiomelanocortin
PRR	proline-rich region
PUMA	p53 upregulated modulator of apoptosis
PVDF	polyvinylidene difluoride
RGP	radial growth phase
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
RTK	receptor tyrosine kinase
SCC	squamous cell carcinoma
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodeyl sulphate polyacrylamide gel electrophoresis
Ser	serine
Smad2, 3	mothers against decapentaplegic homolog 2, 3
SOD	superoxide dismutase
SPARC	secreted protein acidic and rich in cysteine
TAD	transactivation domain
TAFs	TATA - binding protein-associated factors

TBP	TATA - box binding protein
TBS	tris-buffered saline
TET	tetramerization domain
TAFs	TATA box binding protein-associated factors
TGF-β	transforming growth factor-β
TGF-βR	TGF-beta receptor
THI	tyrosine hydroxylase I
TEMED	tetramethylethylenediamine
TNFα	tumour necrosis factor α
TPx	thioredoxin peroxidase
TR	thioredoxin reductase
TRITC	tetramethyl rhodamine isothiocyanate
TRP-1, 2	Tyrosinase related proteins-1 and 2
Tween 20	polyoxyethylenesorbitanmonolaureate
TYR	tyrosinase
Tyr	tyrosine
TβR-I	TGF-β type I receptor
UVR	ultraviolet radiation
UVA	ultraviolet A
UVB	ultraviolet B
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VGP	vertical growth phase
W /W	wild type
Zn	zinc
α-MSH	α-melanocyte stimulating hormone
β –LPH	β -lipotropic hormone
β-END	β-endorphin

Part I

Studies on the p53 / p76^{MDM2}/ MDM4 / SPARC/ VEGF-A and TGFβ 1 cascade in vitiligo

1. Introduction

1.1 Human Epidermis

1.1.1 Structure and function of the skin

The skin represents the largest, complex, multilayered organ of the human body which is constantly renewed and differentiated. It consists of several layers including horny layer, epidermis, basement membrane, dermis and subcutis **(Figure 1)**. The skin represents a biological barrier between external environment and internal organs (Hollbrook and Wolff, 1993; Hall, 2000) protecting the body against physical, thermal and mechanical injuries. Moreover, it hinders body fluids and protein loss and defends against ultra violet radiation (UVR). It regulates body temperature, and sweating in addition to vitamin D synthesis (Graham-Browns and Bourke, 1998; Graham-Brown and Burns, 2011; Hollbrook and Wolff, 1993).

1.1.1.1 Subcutis and Dermis

The subcutis is the innermost layer of the skin which holds nerves and blood vessels. Moreover, it is the part of the skin where storage of fat takes place. It contains the largest adipose tissue volume in the body (Hall, 2000).

The dermis holds a network of connective tissue. This network mainly consists of collagen fibres and some elastin fibres surrounded by a mucopolysaccharide matrix. These fibres are responsible for strength and elasticity (Graham-Browns and Bourke, 1998; Graham-Brown and Burns, 2011). The dermis is rich in blood and lymphatic vessels, nerves, sensory receptors in addition to pilosebacious, eccrine and apocrine tissue. Many types of cells are situated in the dermis including fibroblasts, the cells producing collagen and elastin. In addition, there are e.g. mast cells which play a critical

role during immune and inflammatory responses of the skin by stimulating chemotaxis and phagocytosis, changing vasotension and vascular permeability (Graham-Browns and Bourke, 1998; Graham-Brown and Burns, 2011; Haak et al., 2001). The dermis contains the **papillary dermis** which is located directly beneath the basement membrane. This layer contains thin collagen fibres, **reticular or deep dermis**, holds thick collagen bundles (Hall, 2000).

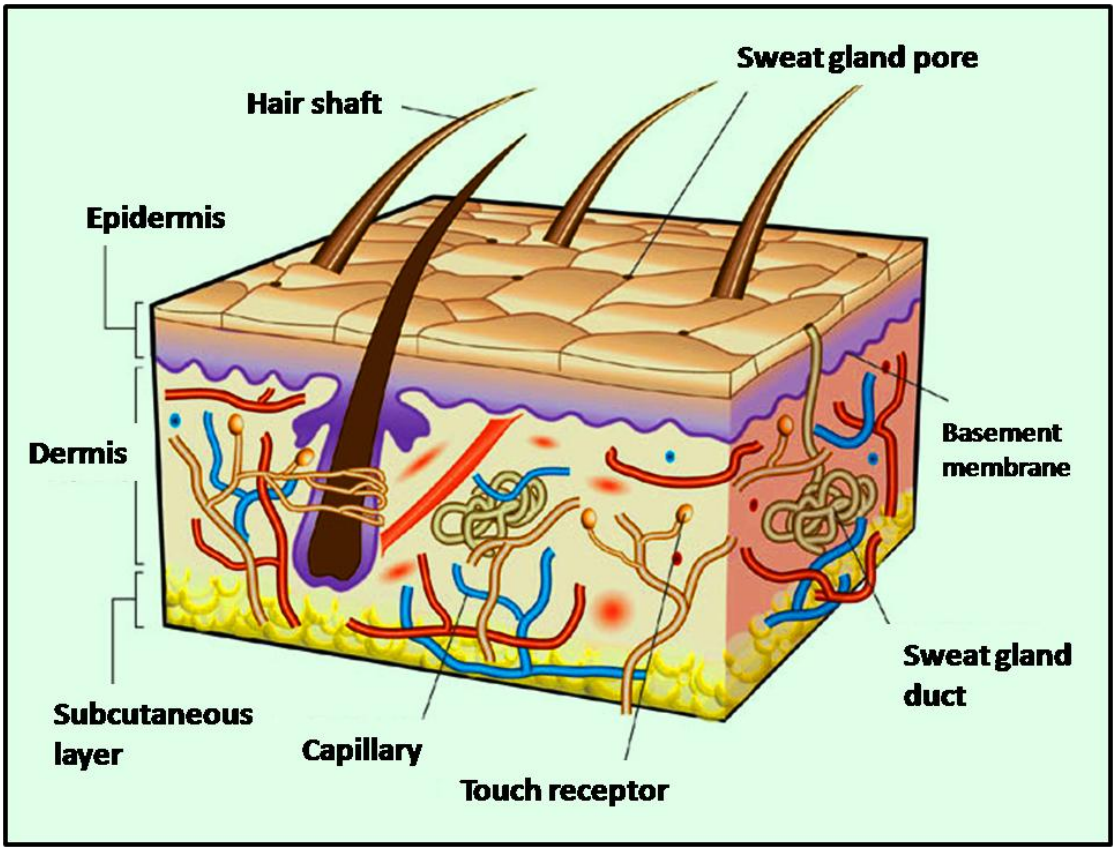

Figure 1: General structure of the human skin.

Skin consists of three main layers: epidermis, dermis and subcutis. Epidermis is a stratified squamous epithelium that extends from basal to superficial layers. Epidermis is separated from the underlying dermis by the basement membrane. The dermis holds a network of connective tissue and is rich in blood and lymphatic vessels, nerves, sensory receptors in addition to pilosebacious, eccrine and apocrine tissue **(from Shier et al., 1999).**

1.1.1.2 The basement membrane zone (BMZ)

The BMZ is a multilayered narrow zone, located between epidermis and dermis playing a crucial role in maintenance of adhesion. (Graham-Browns and Bourke, 1998; Graham-Brown and Burns, 2011) **(Figure 2 shows a schematic view of the BMZ).**

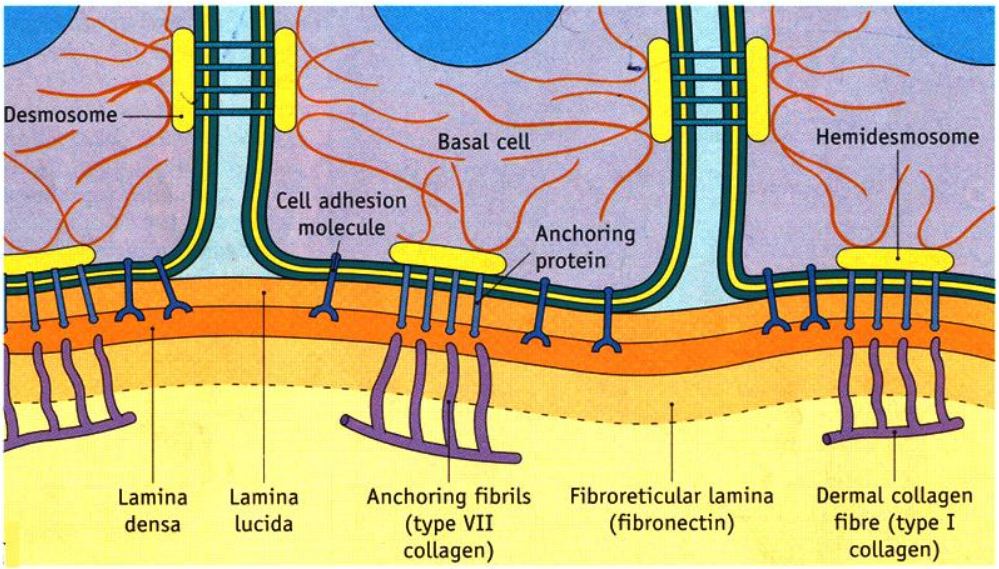

Figure 2: Structure of the basement membrane.

The membrane consists of three layers: lamina fibrorecticularis, lamina densa and lamina lucida. Basal keratinocytes attach to the lamina lucida via hemidesmosomes. Anchoring filaments and anchoring fibrils connect the lamina densa to both hemidesmosomes and dermis respectively **(from Graham-Browns and Bourke, 1998).**

1.1.1.3 The epidermis

The epidermis is formed by a continuously renewed stratified squamous epithelium. It spans from basal to superficial layers holding proliferation and differentiation including keratinisation and cornification (Haak et al., 2001). It can be divided into living layers, i.e. basal layer, spinous layer and granular layer. The stratum corneum contains mainly superficial dead end-products without nuclei (Hall, 2000). The epidermis contains different types of cells, including keratinocytes, melanocytes, Langerhans and Merkel cells. In addition, stem cells are found along the basal lamina border (Lavker and Sun, 1982) **(Figure 3).**

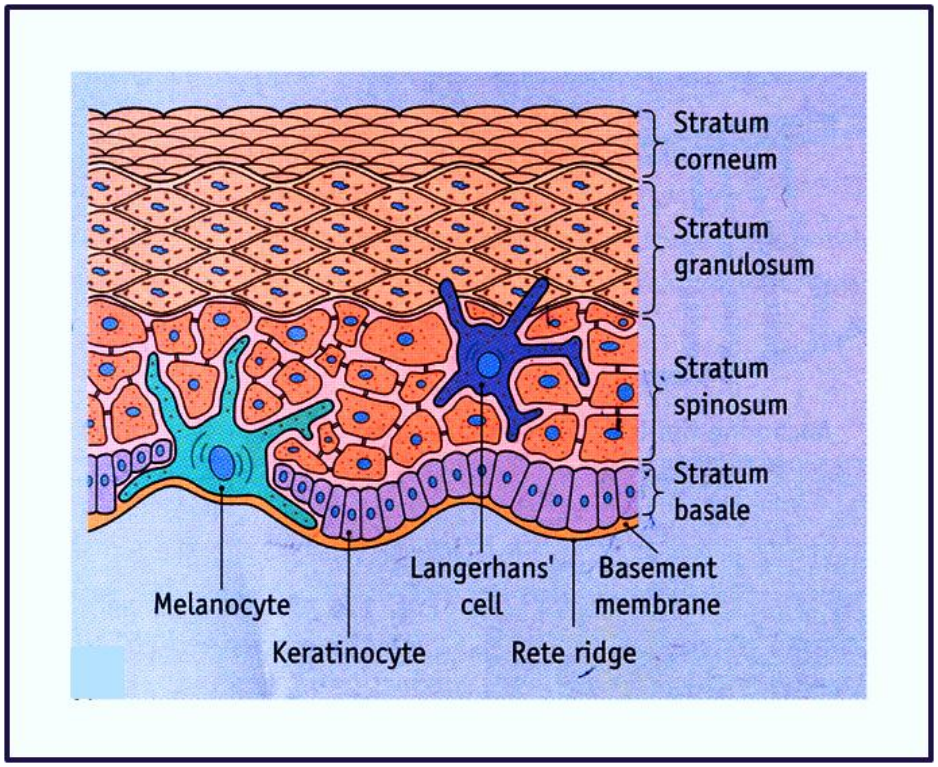

Figure 3: Structure of the epidemis.

The epidermis consists of four distinct layers classified according to location and stage of cell differentiation: the stratum basale attached to the basement membrane, followed by stratum spinosum, stratum granulosum and stratum corneum, the most superficial layer of epidermis (**adapted from Graham-Browns and Bourke, 1998).**

1.1.1.3.1 Keratinocytes

Keratinocytes are keratin forming cells providing the majority of cells in the epidermis, with about 90% of the epidermal cell population. Basal keratinocytes actively divide and differentiate producing different layers including spinous and granular layers which eventually form the stratum corneum with a layer of non-nucleated cells at the surface of the skin. This layer contains mainly keratin. This physiological turnover process takes about 30 days (Graham-Browns and Bourke, 1998; Hollbrook and Wolff, 1993).

Basal cells are columnar in shape with large nuclei and pigmented melanosomes which are transferred from adjacent melanocytes. Those keratinocytes are adhered to the basement membrane via hemidesmosomes. They proliferate approximately every four days, followed by differentiation to generate three to four layers of polyhedral shaped spinous cells with rounded nuclei. These cells are held together by desmosomes which appear ultra-structurally as spines. Gradually, they show more signs for differentiation e.g. an increase of keratin granules content in addition to nuclei disintegration, forming in turn an upper layer of flattened granular cells. Odland bodies or membrane-coating granules provide a thin film of lipids covering the surface of keratinocytes of the cornified layer yielding waterproof and cohesion. N.B. 10 % of the stratum corneum weight is lipids (Elias and Feingold, 1992; Feingold et al., 1990; Feingold, 2007; Graham-Browns and Bourke, 1998; Hollbrook and Wolff, 1993; Sevilla et al., 2007).

Keratinocytes live in symbiotic relationship with melanocytes, regulating melanocyte proliferation, migration, survival and differentiation via releasing several ligands which bind to melanocyte receptors (Abdel-Naser, 1999). In this context, it is noteworthy that keratinocytes hold extensive endocrine function, including production of pro-opiomelanocortin (POMC) derived peptides such as α-melanocyte stimulating hormone

(α-MSH), β-endorphin and ACTH, to name a few. However, here it should be noted that melanocytes produce also POMC-peptides in an autocrine manner (Yaar and Gilchrest, 1991; Slominski et al., 1993; Kunisada et al., 2001; Kauser et al., 2004; Slominski et al., 2004; Haass and Herlyn, 2005).

1.1.1.3.2 Langerhans cells and Merkel cells

Langerhans cells are bone marrow derived mononuclear dendritic cells present in the stratum spinosum. They represent 2 to 8 % of the epidermal cell population and are considered as members of the immune system (Hollbrook and Wolff, 1993; Graham-Browns and Bourke, 1998). Merkel cells are basal layer located cells. Ultra-structurally they contain a dense core of neuro-secretory granules. Moreover, it is thought that Merkel cells act as specialized epidermal nerve endings (Graham-Browns and Bourke, 1998; Hall, 2000).

1.1.1.3.3 Melanocytes

Melanocytes are dendritic melanin-producing cells, deriving from the neural crest. The first description goes back to 1819 by Sangiovanni, who detected chromatophores in squid (Sangiovanni, 1819), while the first description of human melanocytes was by Henle in 1837 (Henle, 1837). These cells are found in the skin, hair matrix, central nervous system, around blood vessels, inner ear, mucous membranes, peripheral nerves of the sympathetic chain as well as in the heart. The epidermal melanocytes are by far the most studied ones amongst them. These cells are located in the basal layer of the epidermis and differ in number, depending on the body location (Cramer, 1991). They are attached to the basement membrane, where they remain in contact with surrounding keratinocytes (Burns et al., 2004; Haass and Herlyn, 2005). Each melanocyte is connected approximately to 36 kerationocytes via its dendrites forming the epidermal

melanin unit (Fitzpatrick and Breathnach 1963; Fitzpatrick, Miyamoto et al. 1967). Surrounding keratinocytes can supply melanocytes e.g. with ligands which can bind to MC receptors affecting in turn proliferation, migration, survival and differentiation. At the same time melanocytes transport melanosomes, the melanin-laden organelles, to surrounding keratinocytes (Slominski, et al., 1993; Kunisada, et al., 2001; Slominski, et al., 2004; Haass and Herlyn, 2005).

1.1.1.3.3.1 Origin, migration and differentiation of melanocytes

As mentioned above, melanocytes are neural crest derived cells. They originate from the neural crest in the second month of gestation as melanoblasts, which migrate dorso-laterally from the head region towards the skin, where they differentiate into mature melanocytes in the epidermis, dermis and hair follicles. Stem cell factor (SCF) and its melanocyte receptor, receptor tyrosine kinase (RTK) c-Kit, are playing a crucial role in the migration of melanocytes into the epidermis and their survival (Holbrook, 1998; Yoshida, et al., 2001). C-kit gene mutation results in hypopigmentation due to the absence of melanocytes migration appearing in the skin as hypopigmented patches (Fleischman, et al., 1991). This is the case in Piebaldism and in Waardenburg syndrome. Moreover, Wnt5a/Frizzled and its melanocyte receptor, Frizzled-5 receptor, were also found to be important for migration of melanocytes. It was shown that overexpression of the ligand related to the increase in motility and invasion in melanomas (Christiansen, et al., 2000; Weeraratna, et al., 2002; Bachmann, et al 2005). The number of melanocytes starts to increase in the dermis between weeks 10 and 12, arriving in the epidermis between weeks 12 and 14. However, they have also been detected in the epidermis between weeks 8 and 10 (Sagebiel and Odland, 1972).

Some melanoblasts may differentiate into melanocytes before entering the epidermis in response to some growth factors e.g. endothelium 3 (Yoshida, 1996). Undifferentiated melanoblasts are able to proliferate and differentiate to epidermal and to hair follicle melanocytes, where some of them depart from the epidermis towards the dermis developing hair follicles (Yoshida, 1996).

1.1.1.3.3.2 Melanogenesis

1.1.1.3.3.2.1 Melanosome biogenesis and transportation

Melanosomes are the cellular sites for melanin production, storage and transport. They are lysosome-related organelles. Melanosomes originate from endosomes (Raposo and Marks, 2007). Biogenesis of melanosomes takes place in four distinct stages **(Figure 4)**. Stage I (pre-melanosomes; non-pigmented endosomes). They contain PMEL17 protein, a melanosomal protein present on the outer surface membrane of pre-melanosomes. Thereafter it penetrates to the intralumenal vesicles (ILVs), where it breaks down forming fibrillar striations. Pre-melanosomes also have a segmental clatherin coat which may be implicated in the entrance of PMEL17 to the ILVs (Clague, 2002; Theos et al., 2006; Raposo and Marks, 2007; Valencia et al 2007; Harper et al., 2008). In stage II the organelle becomes more elongated with more fibrillar striations. At the same time the melanogenic enzymes, tyrosinase and TRP-1, originating from the Golgi apparatus, are transferred to stage II melanosomes either via transport vesicles or via early endosomal intermediates. They start melanin production on the fibrillar striations, leading to transformation of stage II to stage III melanosomes (Hearing, 2005; Raposo and Marks, 2007). The limiting membrane of tyrosinase and TRP-1 positive early endosomes have adaptor complex proteins (AP1 or AP3) coated buds. These adaptor proteins are playing a critical role in the transportation of tyrosinase and TRP-1 to melanosomes (Huizing, et

al., 2001; Theos, et al., 2005). Moreover, it was found that other protein complexes such as BLOC1 and BLOC2 are involved in transport of melanogenic enzymes from endosomes to melanosomes. It was shown that lack of each of them leads to accumulation of TRP-1 in early melanosomes (Di Pietro, et al., 2006; Setty, et al., 2007). With increased melanin production in stage III melanosomes, the organelles become dense, forming fully matured stage IV melanosomes (Raposo and Marks,2007). Mature melanosomes are classified into eumelanosomes and pheomelanosomes. **Eumelanosomes** (electron-dense melanosomes) are about 1µm in length, ellipsoidal with a network of intramelanosomal fibrillar striations. They also contain glycoprotein matrix, which is important for eumelanin biosynthesis. They are mainly distributed singularly. **Pheomelanosomes** are small, variable in shape, but probably spherical with a vesicular matrix and less glycoprotein matrix. They aggregate in membrane bound complexes within keratinocytes (Imokawa and Mishima, 1986).

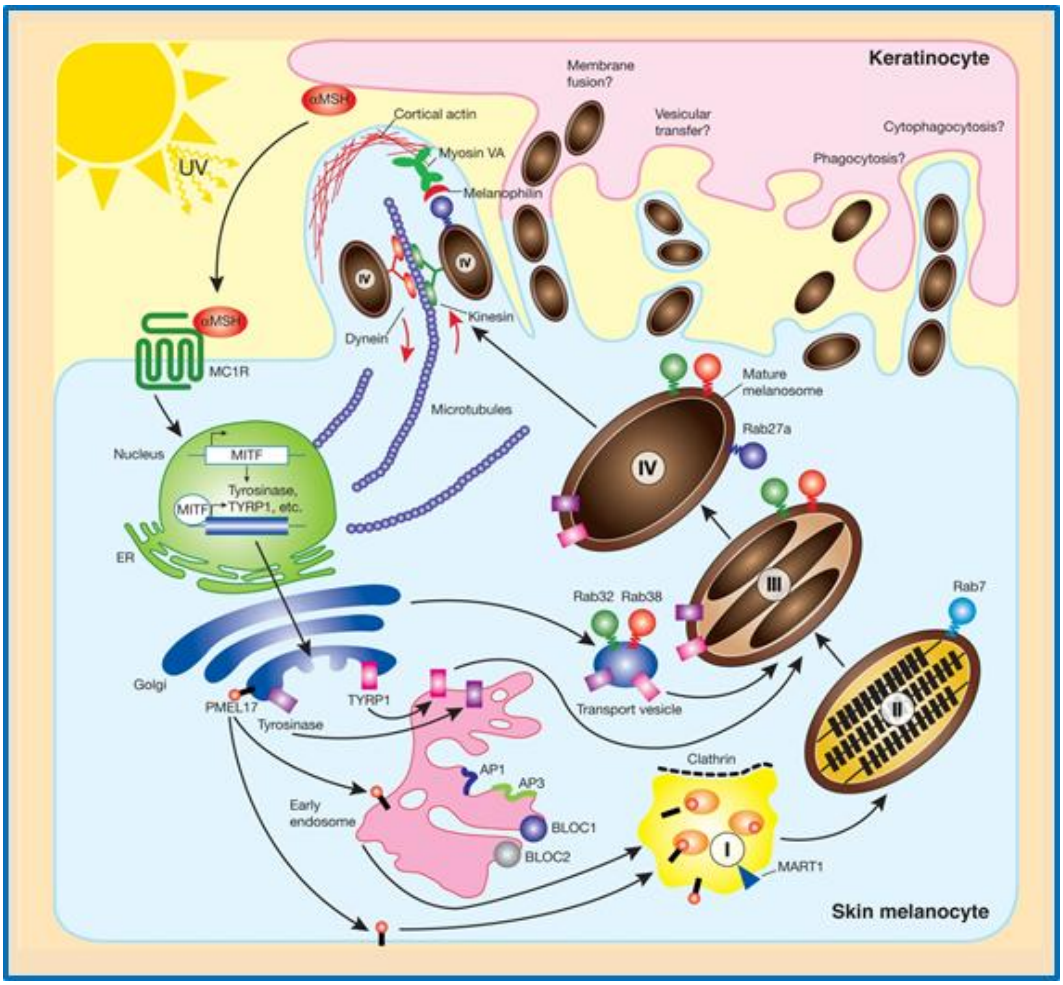

Figure 4: Biogenesis of melanosomal maturation stages and their transportation to keratinocytes.

In Stage I, PMEL17 protein penetrates to the intralumenal vesicles (ILVs) of pre-melanosomes. In stage II the organelle becomes more elongated with more fibrillar striations. Deposition of melanin on this fibrillar striations leads to the transformation of stage II melanosomes to stage III melanosomes. The limiting membranes of tyrosinase and TRP-1 positive early endosomes have AP1 or AP3 coated buds. With the increase in melanin production in stage III melanosomes, the organelles become dense and the cellular architecture is no longer detectable. Mature melanosomes transport from the perinuclear region to the dendrites via microtubule (MT)-dependent long-distance transport of melanosomes. They detach from microtubules after binding of GTPase Rab27a and its MLph to MYO5A in turn enhancing the binding of melanosomes to the cortical actin **(from Wasmeier et al., 2008).**

Mature melanosomal transport starts from the perinuclear region to the dendrites. The first step involves the microtubule (MT)-dependent long-distance transport by kinesin and dynein motors to the dendrite tips. The second step involves the detachment of melanosomes from microtubules after binding of GTPase RAB27a and its effector melanophilin (MLph) which acts as melanosomes-associated receptor to myosin 5A (MYO5A (Wu et al., 1998; Hammer and Wu, 2002; Seabra and Coudrier, 2004). Several theories have been proposed for melanosomal transfer from melanocytes to keratinocytes (for review see Van den Bossche, et al., 2006):

1- Cytophagocytosis of melanin loaded tips of melanocytic dendrites by its neighboring keratinocytes (Okazaki et al., 1976; Cruickshank and Harcourt, 1994; Yamamoto and Bhawan, 1994).

2- Exocytosis that involve fusion of melanosomal membrane with melanocyte plasma membrane releasing melanin to the extracellular matrix, followed by engulfment from keratinocytes (Swift, 1964; Yamamoto and Bhawan, 1994).

3- Transfer by membrane-bound vesicles (Cerdan et al., 1992).

4- Filopodia dependent melanosomal transfer from melanocytes to keratinocytes (Scott et al., 2002; Singh, et al., 2010).

1.1.1.3.3.2.2 Regulation of melanogenesis

Melanins are the final product of melanogenesis. They are large multimorphous biopolymers. Both eumelanin and pheomelanin are resulting from oxidation of L-tyrosine to L-DOPA in the presence or absence of sulfhydryl groups from cysteine. Eumelanin (black) derives from oxidative polymerization of 5, 6-dihyroxyindoles, while pheomelanin (yellow-red) derives from oxidative polymerization of cystein-S-YL dopas and containing in addition to nitrogen some sulphur groups (Prota et al., 1998; Ito, 2003) (see melanogenesis pathway in **Figure 5)**. L-tyrosine is transported through plasma membrane via facilitated diffusion rather than active transport (Schallreuter and Wood 1999c). Because of too low tyrosine levels inthe periphery, facilitated diffusion seems to be insufficient for melanogenesis (Schallreuter and Wood 1999c; Schallreuter et al., 2005c). In this context it has been recognised that tyrosine is formed intra-cellularly in melanocytes via hydroxylation of L-phenylalanine by the action of intracellular phenylalanine hydroxylase (PAH) (Embden and Baldes 1913; Schallreuter et al., 2005c) in the presence of the cofactor (6R)-L-erythro 5, 6, 7, 8 tetrahydrobiopterin ($6BH_4$). The cofactor reduces the iron in the catalytic region of PAH from the inactive ferric state to the active ferrous form (Kaufman 1957; Kaufman 1958; Kaufman 1959; Schallreuter and Wood 1999c). Calcium controls the active transport of L-phenylalanine into melanocytes and its turnover to L-tyrosine via a calmodulin dependent Ca^{2+}-ATPase (Schallreuter and Wood, 1999c; Schallreuter et al., 2005c)

The second step includes production of 3, 4-dihydroxyphenylalanine (L-DOPA) in the presence of tyrosine hydroxylase I (THI) again in the presence of the co-factor $6BH_4$. Once tyrosinase is activated to met-tyrosinase via a 2-electron reduction by THI, the enzyme can oxidize L-tyrosine to L-DOPA (Wood et al., 1995; Olivares et al., 2002; Marles et al., 2003). Here it is noteworthy that the cofactor $6BH_4$ is also required

regulation of tyrosinase. (Kaufman, 1997; Marles et al., 2003; Pey et al., 2006). $6BH_4$ is an allosteric inhibitor of tyrosinase, only when L-tyrosine is the substrate (Wood et al., 1995).This result showed that tyrosinase has 2 distinct binding sites, one for L-tyrosine and the other for L-DOPA (Wood et al., 1995; Olivares et al., 2002).

Melanogenesis can be affected by many factors. These factors can originate from paracrine and endocrine sources or by the external environment including ultraviolet radiation. These signals are targeting MITF amongst many others (Lin and Fischer, 2007). MITF is a transcription factor for several genes that are implicated in melanocyte development and melanosomal biogenesis (tyrosinase, TRP-1, TRP-2, tryptophan hydroxylase (TPH), PMEL17, OA1 and Rab27) (Levy et al., 2006; Chiaverini et al., 2008; Cheli et al., 2009, Schallreuter et al., 2012a).

Melanocortins are derivatives of the precursor hormone proopiomelanocortin (POMC) and play crucial a role in melanogenesis via binding to melanocortin-1 receptor (MC1 - R) (Eipper and Mains, 1980; Lin and Fisher, 2007). POMC gives rise to many bioactive peptides including α-melanocyte stimulating hormone (α -MSH), β-MSH, β-lipotropic hormone (β -LPH), adrenocorticotropic hormone (ACTH) and β-endorphin (β-END) via cleavage by pro-hormone convertase 1 and 2 (Benjannet et al., 1991; Seidah et al., 1994). Notably, the MC4 - R has been implicated in regulation of pigmentation via β-MSH (Spencer and Schallreuter, 2009).

UVR enhances the production of H_2O_2 which in turn increases p53 via NFκB (Vile, 1997; Xie et al., 1999). These observations suggested a role for H_2O_2 in skin pigmentation via a p53 dependent pathway. It was demonstrated that p53 up-regulation enhances the POMC machinery specifically through α-MSH⁄MCR-1 (Cui et al., 2007).

A regulatory role for low (10^{-6} M) H_2O_2-levels has been shown on melanin synthesis via activation of tyrosinase (Wood et al., 2004). However, H_2O_2 in millimolar

concentrations, as it exists in vitiligo, can oxidise many proteins and peptides, including tyrosinase (Schallreuter et al., 2000a; Schallreuter, 2014) and the prohormone convertases PC1, PC2, Furin, PACE4 as well as the POMC-derived peptides α-MSH and β-endorphin (Spencer et al., 2007). Furthermore, H_2O_2 oxidises methionine residues to methionine sulfoxide in the active site of tyrosinase in position 374 leading in turn to its deactivation (Wood et al., 2004; Wood et al., 2005, Wood et al 2008). It has been shown that H_2O_2 can oxidise the calcium binding protein calmodulin, affecting in turn calmodulin dependent Ca^{2+}-ATPase of L-phenylalanine uptake by melanocytes which in turn would hamper L-tyrosine supply for melanogegesis (Schallreuter and Wood, 1999c; Schallreuter et al., 2004b; Schallreuter et al., 2007c).

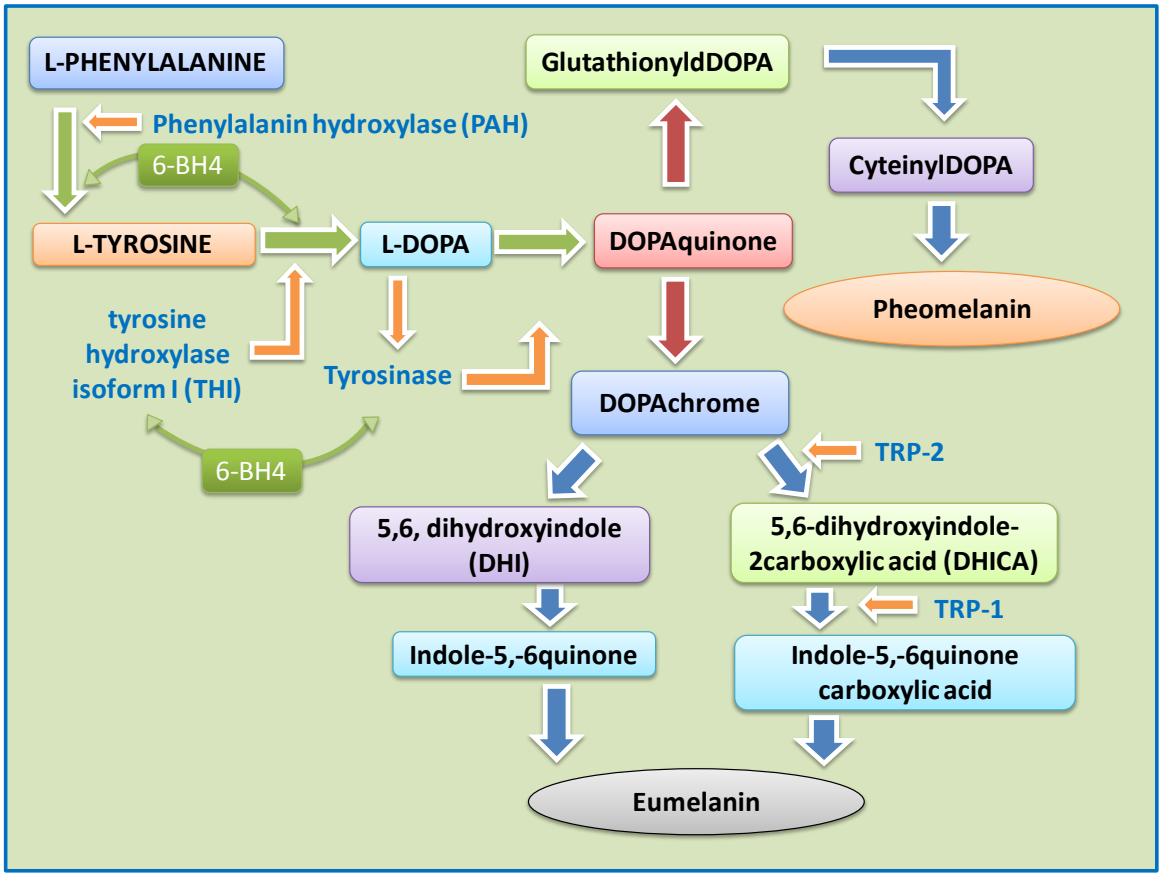

Figure 5: Scheme of melanogenesis.

Eumelanin and pheomelanin biosynthesis is starting with uptake and hydroxylation of L-phenylalanine to L-tyrosine. L-tyrosine is catalysed by THI to 3,4-dihydroxyphenylalanine (L-DOPA) (Schallreuter and Wood, 1999c; Schallreuter et al, 2004b). $6BH_4$ regulates both enzymes, PAH and tyrosinase (Wood et al., 1995: Schallreuter and Wood, 1999c, Schallreuter 2014). After activation of tyrosinase by THI, the enzyme catalyses the oxidation of L-DOPA to dopaquinone. At this point eumelogenesis and pheomelanogenesis pursue two different pathways. In eumelanogensis, cyclization of dopaquinone results in formation of cyclodopa (leukodopachrome). Oxidation of cyclodopa gives rise to formation of a red compound called dopachrome which becomes rearranged to form 5,6, dihydroxyindole (DHI) or 5,6-dihydroxyindole-2carboxylic acid (DHICA) which are polymerized to produce eumelanin (Pawelek,1990; Prota, 1992; Prota, 1995; Ito, 2003). In pheomelanogensis, via non enzymatic reactions dopaquinone gains one SH group from cysteine to form cysteinyl dopa which has two forms, 2-cysteine-S-YL-dopa (2-cysdopa) and 5-cysteine-S-YL-dopa (5-cysdopa). Furthermore, dopaquinone can also bind to gluathione forming glutathione dopa (colourless product) which in turn can be transferred to cysteinyldopa forming pheomelanin (Prota, 1992; Prota, 1995; Ito, 2003).

1.2 Vitiligo

1.2.1 Definition and epidemiology of vitiligo

The name vitiligo originates from the Latin words, vitium = anomaly or vitelius = white patches on calf's fur (Kovacs, 1998; Pavlović et al., 2000; Ortonne et. al., 2003). Vitiligo is an idiopathic, acquired and non-contagious skin disorder, characterised by loss of pigmentation due to either complete absence of melanocytes or loss of their functionality, leading in turn to white patches with different sizes, shape and locations. These days it is without any doubt that keratinocytes and Langerhans cells are also part of the game (Nordlund and Ortonne, 1992; Le Poole et al., 1993, Ortonne and Bose, 1993; Passi et al., 1998; Tobin et al., 2000; for review see Schallreuter et al 2008a, Schallreuter 2014).

Wood`s light examination is used for correct diagnosis of vitiligo as it helps distinguishing between vitiligo and other leukodermas, based on the fact that vitiligo lesions show a distinct fluorescence when exposed to Wood`s light (UVA 351nm). This fluorescence is due to the presence of oxidised pterins which are absent in other skin depigmentations (Schallreuter et al., 1994a,b).

According to the world health organization, vitiligo is a disease which is considered as a common disorder of skin pigmentation. Its prevalence is ranging between 0.5 and 1% worldwide (Alkhateeb et al., 2003; Parsad et al., 2003; Daneshpazhooh et al., 2006; Moretti et al., 2006; Sehgal and Srivastava, 2007; Wolff et al., 2007; Szczurko and Boon, 2008; Lotti et al., 2008; Krüger and Schallreuter, 2012) regardless of gender, skin color and age (Lebwohl et al., 2006; Moretti et al., 2006; Lotti et al., 2008 a;b; Wolff et al., 2007; Schallreuter 2014).

Different studies have reported a predominance for childhood or young adulthood, where vitiligo seems to appear with a peak between10-30 years (Tonsi, 2004; James et al., 2006; Wolff et. al., 2007). However, it can develop at any age (Behl and Bhatia, 1971; Mehta et al., 1973; Halder and Nootheti, 2003; Wolff et. al., 2007; Schallreuter et al 2008b; Szczurko and Boon, 2008). Vitiligo is rare in older people and infants and its rate seems to be declining with age (Tonsi, 2004; Lotti et al., 2008a; b).

1.2.2 Diagnosis and clinical classification of vitiligo

Vitiligo lesions are diagnosed as chalk- or milk- white patches that can occur at any place of the body of healthy individuals including face the dorsal surface of hands, sacrum, axillae, nipples, umbilicus and inguinal / anogenital regions as well as flexor wrists, digits, knees and elbows (El-Din Anbar et al., 2008; Alikhan et al., 2011). As said above, correct diagnosis is made by Wood`s light (351 nm) (Schallreuter et al., 1999a;b). According to distribution and location of the white spots, the disease is classified.

1.2.2.1 Localized vitiligo

It is characterized by the presence of few white macules. It can be subtyped into segmental and focal (Hann and Im 2004; Gawkroger et al., 2008).

1.2.2.1.1 Segmental vitiligo (dermatomal and /or Blasckolinear)

Unilateral vitiligo is characterized by an appearance of depigmented skin on one side of the body (Hann and Lee, 1996; Hann, 2000c). Clinically the depigmentation can affect one or several dermatomes or Blaschko lines (Schallreuter et al, 2007e). Accordig to the literature it affects children more than adults (Koga and Tango, 1988; Hann and Lee, 1996; Grimes and Billips, 2000; Hann and Im, 2004). After the onset of the

depigmentation, it mostly takes 1 to 2 years to spread, but then it usually stops. It is rare that unilateral vitiligo patients develop bilateral vitiligo (Koga and Tango, 1988; Hann and Lee, 1996; Hann and Im, 2004), leading to **mixed vitiligo.** The latter entity has been surprisingly missed over decades (Schallreuter et al., 2008b; Ezzedine et al., 2012).

1.2.2.1.2 Focal vitiligo

Focal vitiligo refers to depigmentation of skin in one or more patches with random distribution (Nordlund and Lerner, 1982; Lotti et al., 2008b). In some patients these patches remain localized, while in other less fortunate cases, it develops to more aggressive subtypes such as vitiligo vulgaris (Liu et al., 2005).

1.2.2.2 Generalized vitiligo

Generalized vitiligo is considered to be the most common type of vitiligo that can be divided into two subtypes namely acrofacial and vitiligo vulgaris. Recently a new classification has been suggested by the European Task Force group (Taïeb and Picardo, 2007). For generalised vitiligo the term non-segmental vitiligo (NSV) has been introduced.

1.2.2.2.1 Acrofacial vitiligo

This term refers to depigmented patches of skin that start to appear on distal extremities (fingers and feet) and acrofacial areas (around mouth and eyes) including the perianal and genital area. Patients of this type of vitiligo represent 3 to 16 % of all vitiligo cases (Schallreuter et al., 1994c; Parsad et al., 2004; Zhang et al., 2004; Dogra et al., 2005; Liu et al., 2005; Nordlund et al., 2008).

1.2.2.2.2 Vitiligo vulgaris

The most common subtype of vitiligo shows symmetrical appearance of large white macules anywhere on the entire integument (Nordlund et al., 2008; Gawkrodger et al., 2008; Arycan et al., 2008; Halder and Taliaferro, 2008). It represents about 40 -80% of all vitiligo cases (Handa and Kaur, 1999; Dogra et al., 2005; Liu et al., 2005).

When vitiligo spreads involving >80% of the skin, it is described as universal vitiligo (also vitiligo totalis) (Shajil et al., 2006; Nordlund and Lerner, 1982).

1.2.2.3 Vitiligo with unusual patterns

1.2.2.3.1. Vitiligo ponctuè (guttate vitiligo)

It is a frequent type of vitiligo appearing as confetti-like, separated, tiny depigmented macules spread on normal or hypermelanotic skin. The prevalence increases with age (Ortonne, 2008). Whether this entity presents classical vitiligo is still under debate.

1.2.2.3.2 Trichrome and quadrichrome vitiligo

Trichrome vitiligo patients show 3 different skin color patterns. The hypopigmented area has higher number of melanocytes, compared to amelanotic lesions, but lower numbers than non-lesional skin. Moreover, it shows an increase in the number of Langerhans cells, inflammatory cells and melanophages in comparison with both lesional and non-lesional skin of these patients (Lerner, 1959; Fitzpatrick, 1964; Hann et al., 2000b).

1.2.2.3.3 Blue vitiligo (pentachrome vitiligo)

It shows additional blue-grey macules that may be due to epidermal melanocyte absence. These patients are supposed to have melanophages in their dermis (Chandrashekar, 2009).

1.2.2.3.4 Inflammatory vitiligo

This type of vitiligo exhibits raised red borders around the depigmented macules which may be accompanied with mild pruritus due to irritation caused by an inflammatory process and lymphocytic/histocytic infiltration (Fitzpatrick and Mihm, 1971; Hann and Im, 2004; Sharquie et al., 2004; Halder and Taliaferro, 2008; Ortonne, 2008). Recently pruritus in vitiligo has been attributed to extreme oxidative stress in acute vitiligo (Schallreuter, 2014).

1.2.2.3.5 Blaschko-linear vitiligo

Blaschko-linear vitiligo is a peculiar depigmentation following Blaschko`s lines. Those lines were first described and delineated in 1901 by Alfred Blaschko (Blaschko, 1901). In this case depigmentation shows fountain-like lines on the chest/abdomen and perpendicular lines on face and limbs (Jakson, 1976; Happle, 1977; Bolognia et al., 1994;).

Although Blaschko-linear pattern is rare in the literature (El Mofty, 1968; Punshi, 1975; Schallreuter et al., 2007e), some studies claim its occurrence in a fairly high rate (49.6%) in segmental vitiligo (Khaitan et al., 2007).

1.2.3 Etiology of vitiligo

The cause of this disease is still unknown. Several theories have been evolved, reaching from destruction of functioning melanocytes to complete loss with subsequent appearance of vitiligo macules.

1.2.3.1 Autoimmune hypothesis

This theory involves implication of autoimmune response for melanocyte destruction and pathogenesis of vitiligo and how components of the autoimmune system, including memory cytotoxic T-cells or autoantibodies are directed against surface antigens of melanocytes in addition to the presence of thyroid, parietal mucosa and adrenal antibodies (Naughton et al., 1983; Ongenae et al., 2003; Farrokhi et al., 2005; Le Poole and Luiten 2008; Schallreuter et al., 2008a; Schallreuter et al., 2012 b).

1.2.3.2 Neural hypothesis

As melanocytes are derived from neural crest during embryogenesis, it was proposed that vitiligo represents an abnormal interaction between neurochemical mediators such as neuropeptides including catecholamines and / or their metabolites with melanocytes (Schallreuter et al., 1994b; Orecchia, 2000).

An elevated level of catecholamines and its metabolites have indeed been reported in urine of vitiligo patients and levels were proportionally increasing in association with disease activity (Morrone et al., 1992; Cucchi et al., 2003). Low epidermal phenylethanolamine-N-methyl transferase (PNMT) levels in vitiligo correspond to high norepinephrine levels, suggesting involvement of this catecholamine (Schallreuter et al., 1994b. In this context it is of note that higher expression of epidermal β2-adreno-

receptors has been documented in vitiligo (Schallreuter et al., 1993). Moreover, the entire epidermal cholinergic system is affected in these patients (Schallreuter and Elwary, 2007a). It was also reported that levels of acetylecholine are up-regulated in vitiligo due to loss of functioning regulatory enzymes including AchE (acetylcholinesterase) and BchE (butyrylcholinesterase) (Schallreuter et al. 1996a; Schallreuter et al., 2004a; Schallreuter et al., 2007b; Glassman, 2011). Regulatory factors for melanogenesis, derived from pro-opiomelanocortin (POMC), are affected in vitiligo (Peters et al., 2000). These peptides involve α-MSH (α-melanocyte-stimulating hormone) and β-endorphin (Graham et al., 1999; Spencer et al., 2005; Spencer et al., 2007).

1.2.3.3 Autocytotoxic hypothesis

During melanogenesis some toxic products, such as phenols or quinones, are produced and their accumulation may lead to melanocyte damage (Hann and Chun, 2000). L-tyrosine is a phenol that oxidizes to L-dopaquinone during melanin synthesis. Oxidation of L-dopaquinone releases several phenolic compounds (free radicals or indoles) that can harm melanocytes (Boissy and Manga, 2004). In this context it is of interest that patients with vitiligo are extremely sensitive to those compounds, affecting even lymphocytes (Schallreuter et al., 2006; Schallreuter and Salem, 2010). Moreover, the Slominiski group suggested in 1989 post-tyrosinase inhibition of melanin production due to activation of melatonin receptors causing accumulation of toxic intermediates and free radical accumulation in melanocytes which in turn would lead to its destruction (Slominiski et al., 1989). However, very recently it was shown that the entire melatoninergic as well as the serotoninergic pathway are severely affected in vitiligo leading to absence and /or low levels of melatonin and serotonin. Accordingly there

would not be any ligand available for melatonin and / or serotonin receptors (Schallreuter et al 2012 a).

1.2.3.4 Genetic hypothesis

Many familial cases of vitiligo were documented ranging 1.5- 45%.incidence rates. This fact prompted many studies to suggest the implication of genetic abnormalities in vitiligo (Singh et al., 1985; Schallreuter et al., 1994b; Alkhawajah, 1997). Genetically, vitiligo is defined as an inherited disorder with non-Mendelian, multifactorial and multi genetic pattern, with incomplete penetrance (Alkhateeb et al., 2003; Sun et al., 2006).

Some studies reported aberrant expression of melanocyte specific proteins including c-Kit and stem cell factor (SCF). Both represent an important receptor/ligand system in regulation of melanocyte differentiation and melanogenesis (Chabot et al., 1988; Zsebo et al., 1990). Moreover, the catalase gene was documented, to be affected by several point mutations. This observation can support both, a genetic and oxidative stress hypothesis (Casp et al., 2002; Wood et al., 2008).

Recently two large-scale genomewide association studies (GWAS) of generalized vitiligo (GV), one in Caucasians and the other in a Chinese population, were reported. At least 16 different genetic loci were associated with susceptibility in generalized vitiligo. All of these genes, except one, are encoding proteins that are part of the immune system regulation and/or have been genetically associated with susceptibility to other autoimmune diseases. The only identified gene with no association to the immune system was TYR, encoding tyrosinase, the key enzyme of melanin biosynthesis (Jin et al., 2010; Quan et al., 2010). Future work needs to unravel the connection between bench and bedside. Importantly, these novel data need to be brought together with all current and old knowledge on the subject vitiligo.

1.2.3.5 Viral hypothesis

This theory proposes the implication of viral infections in the pathogenesis of vitiligo, as some authors suggested a relationship between this disease and some viruses,including human cytomegalovirus (CMV), hepatitis C virus (HCV) and Epstein Barr virus (EBV) (Grimes et al., 1996; Grimes et al., 1999; Galarza et al., 2004), while other studies could not confirm any significant connection between vitiligo incidence and viral infections (Würfel, 1999; Akbayir et al., 2004; Schallreuter, 2004b; Jadali et al., 2005).

1.2.3.6 Oxidative stress hypothesis

Oxidative stress refers to a disturbance in the cellular redox status in form of an imbalance between production and manifestation of oxygen containing molecules, called reactive oxygen species (ROS) including H_2O_2 and the ability of an effective antioxidant system to promptly detoxify these ROS. Accumulation of these intermediates in the cells may lead to oxidation of cellular components, affecting in turn different pathways, including gene and protein expression in addition to signal transduction (Sies and Cadenas, 1985; Wiseman et al., 1995; Migliore and Coppede, 2002; Stadtman, 2004; Schallreuter, 1999b, 2005a, 2008a; 2014). ROS lead to production of free radicals, which are unstable molecules owning unpaired electrons in an orbit such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and $ONOO^-$ to name a few (Sies and Cadenas, 1985; Cobbs et al., 1995; Cobbs et al., 2001; Migliore and Coppede, 2002,; Schallreuter et al 1999b, 2005a; 2014).

H_2O_2 production in epidermal cells originates from multiple endogenous or exogenous sources. Endogenously it involves activation of various enzymes; including tyrosinase (Wood et al., 2004), monoamine oxidase A (Barzu and Dansoreanu, 1980; Maker et al.,

1981; Schallreuter et al., 1996b, xanthine oxidase (Olson et al. 1974; Kellogg and Fridovich, 1975; Parks and Granger, 1986; Schallreuter et al.,1996b; Shalbaf et al ., 2008) , NADPH oxidase (Rossi et al., 1985), tyrosine hydroxylases (Adams et al., 1997) and the nitric oxide synthases, (Salem et al., 2009) in addition to various growth factors including transforming growth factor-β (TGF-β) (Shibanuma et al., 1991; Thannickal et al., 1993), platelet derived growth factor (PDGF) (Sundaresan et al., 1995), epidermal growth factor (EGF) (Bae et al., 1997) and tumor recrosis factor α (TNFα) (Hoffman and Weinberg, 1987) as well as aromatic steroids such as estrogen (Schallreuter et al., 2006) and progesterones (Martinoli et al., 1984) . Exogenously, phenols, ortho / para quinols in addition to UVA / UVB and X-rays can also contribute to the H_2O_2 production and consequently to the radical pool (Schallreuter et al., 1999b; Schallreuter and Wood, 2001) (**see Figure 6)**.

Superoxide anion radicals ($O_2^{\cdot -}$) can release iron from cellular molecules and this iron takes part in the Fenton reaction producing $^{\cdot}OH^{\cdot}$ from H_2O_2 as shown below (Stohs and Bagchi, 1995; Schallreuter et al., 2012a).

$$\mathbf{Fe\ (II) + H_2O_2 \longrightarrow Fe\ (III) + {}^{\cdot}OH + OH^-}$$

$OH^{\cdot}$ can react with amino acids and fatty acids affecting cell membranes and protein function, enzyme activity and transcription processes (Stohs and Bagchi, 1995; Pekarkova et al., 2001; Liochev and Fridovich, 2002; Leonard et al., 2004; Smith, et al., 2004; Schallreuter et al., 2012a).

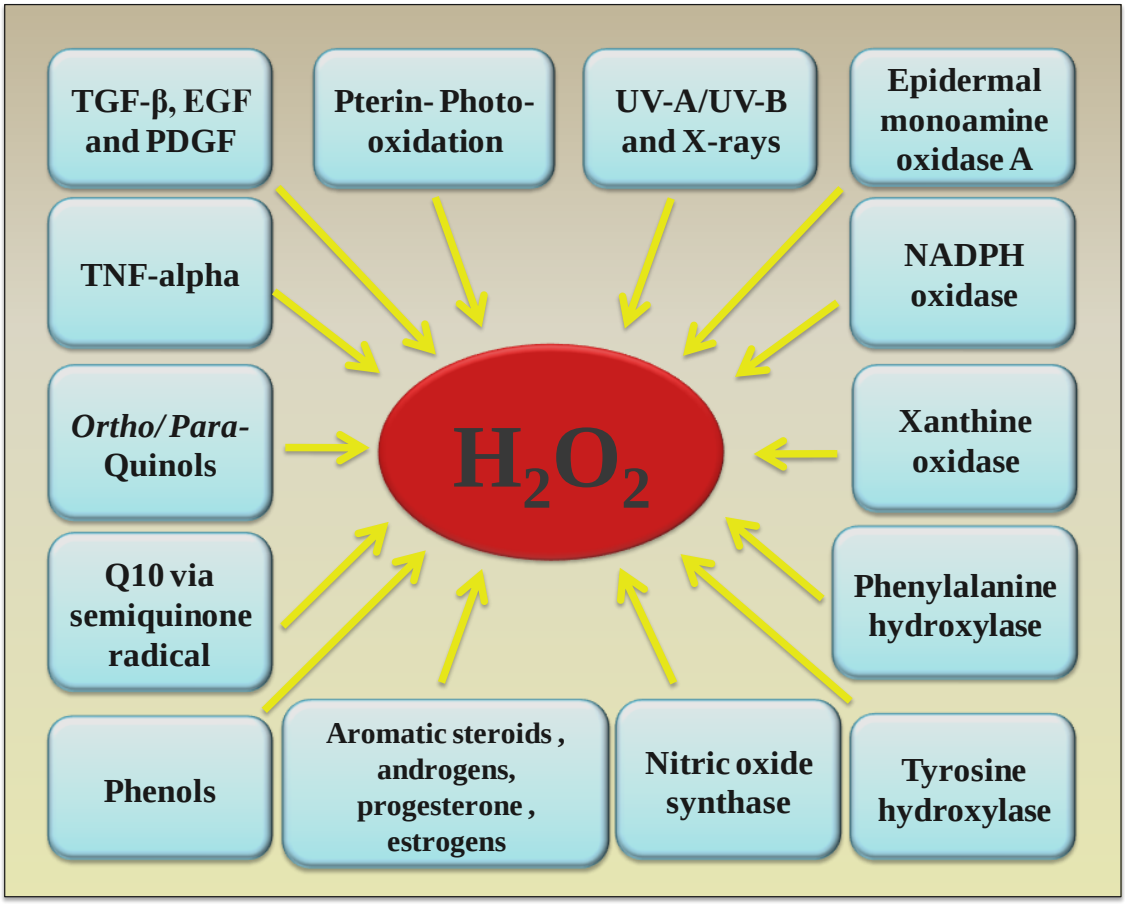

Figure 6: H_2O_2 generation in the human epidermis.

TNF-α indirectly leads to H_2O_2 formation via the induction of manganese superoxide dismutase (Schallreuter, 2005a; Moretti et al, 2002; Wong and Goeddel, 1988). TGF-β, EGF and PDGF were reported to generate H_2O_2 (Thannickal et al., 2000). Epidermal monoamine oxidase A activities generate H_2O_2 (Darr and Fridovich, 1994). NADPH oxidase activities from neutrophils and macrophages generate H_2O_2 (oxygen burst) (Darr and Fridovich, 1994). Photo-oxidation of epidermal $6BH_4$ and sepiapterin yields H_2O_2 generation (Schallreuter, 1999). Nitric oxide synthases can spontaneously foster the synthesis of H_2O_2 in the absence of the substrate L-arginine (Schallreuter, 2005; Schallreuter, 1999). Generation of H_2O_2 by estrogen and progesterone has been demonstrated (Jimbow et al., 2001). Xanthine oxidase degrades purin bases to uric acid and this step generates H_2O_2 (Shalbaf, et al., 2008). Phenols, *o*-and *p*-quinols as well as UVA/UVB and X-rays can generate H_2O_2 in the mM range (Schallreuter et al., 1999b, Boissy and Manga, 2004). Topical application of Q10 generates hydrogen peroxide (H_2O_2) via semiquinone radical formation. Recently we see increasing numbers with the onset of facial vitiligo in susceptible individuals after the use of those Q10 containing cosmetic creams (Schallreuter et al., 2013) **(modified from Schallreuter et al., 2009, 2014).**

In normal skin ROS production is regulated by antioxidant defence mechanisms that involve either enzymes including catalase (CAT), thioredoxin reductase (TR) / thioredoxin , thioredoxin peroxidase (TPx), glutathione reductase(GR) / glutathione, glutathione peroxidase (GPx), superoxide dismutase (SOD) and methionine sulfoxide reductases A and B or non-enzymatic systems such as vitamin C, amino acids as L-methionine, L-cysteine and L-tryptophan in addition to 6- and 7- tetrahydrobiopterins (Schallreuter , 2005a;b; Schallreuter et al., 2008a; Schallreuter, 2014).

In the case of vitiligo, Schallreuter's group were the first to demonstrate *in vivo* a massive increase in H_2O_2 -levels in the epidermis of both lesional and non-lesional skin of those patients, implicating "oxidative stress" via H_2O_2 in the pathogenesis of this disease. This oxidative stress can be measured by the concentration of H_2O_2 or its oxidation products including methionine sulfoxide and other oxidation products by using *in vivo* FT-Raman spectroscopy. Moreover, the epidermal accumulation of H_2O_2 is accompanied with low levels of several epidermal enzymes, which control H_2O_2 concentrations, including CAT, TR and GR, to name a few (Aronoff, 1965; Schallreuter et al., 1991b; Schallreuter et al., 1999b; Schallreuter, 2004a; Chavan et al., 2006; Maresca et al., 2006; Wood and Schallreuter, 2006; Schallreuter et al., 2007 b; c; f; Spencer et al., 2007; Schallreuter and Elwary, 2007a; Wood et al., 2008, Shalbaf et al., 2008; Salem et al., 2009, Schallreuter et al., 2012 a; b; Schallreuter, 2014).

However, in this context it is well established that H_2O_2 in micromolar (10^{-6}M) concentrations has a useful effect on melanogenesis and other important regulatory functions via up-regulating several important transcription factors, including MITF and p53, but also directly on enzyme activities including tyrosinase and some antioxidant enzymes including CAT, TR, GR and methionine sulfoxide reductases (MSR A&B) (Wood et al., 2004; Wood et al., 2005; Schallreuter et al., 2005b; Schallreuter et al.,

2006; Schallreuter et al., 2007c, f; Gibbons et al., 2006; Schallreuter, 2014). Increase of H_2O_2 to the mM range (10^{-3}M) can cause lipid peroxidation and appearance of intracellular vacuolation throughout the entire epidermis in vitiligo patients (Tobin et al., 2000; Bhawan and Bhutani, 1983; Moellmann et al., 1982). Moreover, H_2O_2 can affect melanogenesis negatively via disrupting many of its critical regulatory signals, for example calcium-uptake and its -homeostasis machinery via oxidation of tryptophan or methionine residues of calcium binding proteins including albumin and calmodulin, respectively. The decrease in calcium concentration in melanocytes has a critical effect on melanin production due to the impaired calcium-required active transport of L-phenylalanine and subsequently its turnover into L-tyrosine (Schallreuter and Pittelkow, 1988; Schallreuter et al., 1996a; Schallreuter and Wood, 1999c; Rokos et al., 2004; Schallreuter et al., 2004c). Furthermore it was shown that H_2O_2 influences synthesis and regulation of the essential cofactor $6BH_4$ which can affect melanogenesis via regulating the activity of melanogenic enzymes, including tyrosinase, THI and PAH. H_2O_2 oxidises $6BH_4$ to L-biopterin which is cytotoxic to melanocytes under *in vitro* conditions (Schallreuter et al., 1994d; Schallreuter, 1999a). In fact, as said above, accumulation of oxidised pterines in the skin of patients with vitiligo leads to the characteristic blue / green fluorescence under Wood`s light (351nm) (Schallreuter et al., 1994a,b).

Although H_2O_2 is considered as one of the main players in oxidative stress-mediated vitiligo, other factors are found to contribute to the pathogenesis, such as nitric oxide (NO), a reactive nitrogen species (RNS) member. Its reduction into hydroxyl amine is normally catalyzed by TR, but this enzyme is also deactivated by H_2O_2 in the case of vitiligo. Deactivation of TR leads to accumulation of NO which in turn reacts with $O_2^{\cdot-}$ producing the damaging ROS peroxinitrite ($ONOO^-$) (Salem et al 2009). $ONOO^-$ leads to nitration of tyrosine residues affecting in turn all proteins, which contain this residue

in the structure (van der Vliet et al., 1994; Oury et al., 1995; Groves, 1999). In vitiligo it was shown that epidermal $ONOO^-$ levels are extremely high (Salem et al 2009).

These hazardous effects of high H_2O_2 / $ONOO^-$ concentrations on melanogenesis can be successfully controlled with a topical application of a narrow-band UVB activated pseudocatalase PC-KUS. This approach leads to H_2O_2 removal / reduction by oxidising this ROS back to O_2 and H_2O together with restoration of the missing skin colour (Schallreuter et al., 1995; Schallreuter et al., 2008a; Salem et al, 2009; Schallreuter 2014).

Repigmentation in the face occurs in 95% of all patients. About 80% of them achieved recovery on the integument, while hands / wrists and feet do not respond so well. This clinical result is considered as one important proof for the role of H_2O_2 - mediated oxidation in the pathogenesis of vitiligo (Schallreuter, 1999b; Schallreuter et al., 2008b; Salem et al., 2009; Schallreuter et al., 2012 a; b; Schallreuter 2014).

Moreover, H_2O_2 and $ONOO^-$ - mediated oxidative stress can trigger DNA-damage in patients with this disease. It was recognized that the production of the oxidised DNA base 8-oxoguanine (8-oxoG) is high in both, epidermis and plasma of these patients (Salem et al., 2009). This increase in DNA-damage is associated with epidermal accumulation of H_2O_2 and a functioning wild type p53 in this compartment (Schallreuter et al., 2003; Salem et al., 2009). Importantly, in vitiligo this high p53 is associated with elevated levels of p76 ^{MDM2} splice variant , a competitive antagonist negative regulator of p53 degradation (Perry et al., 2000; Salem et al., 2009).

In this context it was shown that both H_2O_2 / $ONOO^-$ enhance DNA-binding on p53. Consequently it has been suggested that this stable up-regulated p53 accounts for induction of an efficient DNA-repair via hOgg1, APE1 and DNA polymerase β

(Sengupta and Harris, 2005; Salem et al., 2009) that in turn lead to short-patch base-excision repair (BER). Based on this result it was suggested that this cascade may lead to prevention of ROS-induced tumourigenesis in the epidermis of patients with vitiligo (Salem et al., 2009; Schallreuter, 2014).

1.3 Vitiligo and skin cancer

Still the dogma is that melanin is playing a major role in the protection against UVR induced cellular damage by absorbing these rays and transforming them into heat which consequently is dispersed between capillary vessels and hair. It also hinders DNA damage by the action of ROS such as $OH^{\cdot}$ and $O_{2}^{\cdot}$, produced in the skin in response to UVR via scavenging these active metabolites (Hussein, 2005). However, some other studies had reported the deleterious influences of melanin itself via generating ROS after exposure to UVR causing in turn cellular DNA damage (Chedekel et al., 1977; Chedekel et al., 1978; Felix et al., 1978; Chedekel, 1982; Sarna et al., 1985; Korytowski et al., 1987; Prota, 1995; Marrot et al., 1999; Brenner and Hearing, 2008).

Although, vitiligo patients lack protection against UVR due to absence of melanin in the depigmented skin, and hold extremely high ROS-induced oxidative stress in their epidermis due to the accumulation of H_2O_2 and $ONOO^{-}$ accompanied with disturbances in the antioxidant system (Aronoff, 1965; Schallreuter et al., 1991b; Schallreuter et al., 1999 a;b; Schallreuter et al., 2004b; Maresca et al., 2006; Wood and Schallreuter, 2006; Schallreuter et al., 2007a;b;c;d;f ; Wood et al., 2008, Salem et al., 2009; Schallreuter 2014), surprisingly, these patients exhibit no significant elevated risk for skin photo-sensitivity disorders such as polymorphous light reaction, solar urticaria and acute actinic dermatitis or non-melanoma skin cancer such as basal-cell, squamous cell carcinomas and the precursor actinic keratosis. Moreover, their skin appears younger

compared to healthy people of the same age (Oettle, 1963; Calanchini-Postizzi and Frenk, 1987; Schallreuter et al., 2002; Schallreuter et al., 2008a; Teulings et al., 2013; Schallreuter, 2014).

Clearly both, lack of pigment as well as the high ROS-induced oxidative stress, are bad news, leading under normal circumstances to actinic skin damage as well as skin cancer in people with fair-skin and those with certain types of albinism (Kaidbey et al., 1979; Lookingbill et al., 1995; Marks, 1995; Naruse et al., 1997; Salasche, 2000). The crucial question to be answered is: what is the reason behind this enigma and how are patients with vitiligo protected against development of non-melanoma skin cancer?

Schallreuter and colleagues documented *in vivo* and *in vitro* an up-regulated accumulation of H_2O_2-induced functioning wild type p53 throughout the entire epidermis of patients with vitiligo. While reduction of this epidermal ROS with a NB - activated pseudocatalase PC-KUS leads to arrest of the disease as well as to restoration of the skin colour, H_2O_2 -removal / reduction did not affect the high p53 protein level. This observation suggested that H_2O_2 levels in the case of vitiligo may be always high enough for triggering p53 transcription (Vile, 1997; Schallreuter et al., 2003; Schallreuter, 2014). Moreover, these patients hold normal levels of $p90^{MDM2}$, the main negative regulator of p53 (Salem et al., 2009). These findings raised another important question. Why p53 levels are remaining high, if the protein, responsible for its degradation, is not affected as in the case of vitiligo? One explanation could be that those high levels can be attributed to continuous oxidative stress, causing an increase in the post-translational modification of p53 protein in form of phosphorylation in the C-terminal as well as in the N-terminal regions of the protein. These modifications would

destabilize the interaction between p53 and MDM2, resulting in protection of p53 from degradation (Schallreuter et al., 2003, Salem et al 2009).

Salem and colleagues, were the first to shed some more light on the scenario. These authors studied the expression of two splice transcripts of MDM2, namely, p90 ^{MDM2} and p76 ^{MDM2} in the epidermis of patients with vitiligo. They confirmed normal levels of p90 ^{MDM2}, the negative regulator of p53 in association with an up-regulation of p76 ^{MDM2} isomer, a competitive antagonist in binding to p90 ^{MDM2} (Perry et al., 2000). Here it is noteworthy that this variant is not present in normal individuals. The presence of p76 ^{MDM2} could provide an explanation for hindering p53 degradation and accumulation of p53 in the epidermis of these patients (Salem et al., 2009). Furthermore, despite the documented high levels of $ONOO^-$ in these patients and the possibility that it can alter DNA-binding capacity of p53 via nitration of tyrosine residues present in its DNA-binding element, it was shown that p53-DNA binding capacity is enhanced by the presence of both $ONOO^-$ and H_2O_2 in 10^{-3} M concentrations. H_2O_2 is able to abolish the negative effect of $ONOO^-$ via prevention of its ability to nitrate p53 (Salem et al., 2009). Taken together, p53 still seems to be the main master in DNA-repair for patients with vitiligo.

Based on these data it was then proposed that the normal risk of non-melanoma skin cancer in addition to absence of skin photodamage in vitiligo might be connected to this scenario (Calanchini- Postizzi and Frenk, 1987; Schallreuter et al., 2002; Salem et al., 2009).

Although it is now common consent that these patients have no increased risk for development of non-melanoma skin cancer. The data on melanoma in association with vitiligo are controversial (Schallreuter et al., 1991a; Teulings et al., 2013).

Schallreuter and colleagues proposed a 180 - fold higher risk of developing melanoma for patients with vitiligo. The data originated from a study group with 623 patients with melanoma, where 23 had vitiligo and / or melanoma associated leucoderma (MAL). Eleven cases had true vitiligo before melanoma diagnosis, but 11 patients developed depigmentation after diagnosis with primary or / and metastatic melanoma, while in one case first appearance of vitiligo in association with melanoma was not exactly defined (Schallreuter et al., 1991). In this context it is noteworthy that vitiligo was reported as a good prognostic factor for melanoma survival (Boasberg et al., 2006; Cunha et al., 2009, Teulings et al., 2015). This was not the case for those patients in the Hamburg patient group. All patients died within the next 2 years after completion of this study (Schallreuter, unpublished data).

Clearly there is a need to show, whether true vitiligo and /or the presence of MAL is a good prognostic sign for the development of *de novo* melanoma and for prognosis / survival rates. To shed some more light on a possible association / connection with classical vitiligo and MAL is part of this thesis.

1.4 Regulation of p53 by SPARC, TGF-β and the possible involvement of the entire MDM2 family.

1.4.1 p53 – as the "guardian of the genome"

p53 is a transcription factor that can activate or suppress the expression of many genes via binding to their promoter regions (Song and Xu, 2007). In normal cells, p53 is frequently undetectable, as it is expressed in very low concentrations (approximately 1000 molecules / cell) with a short half life (approximately 20 min). It is immediately ubiquitinated by the action of MDM2 and degraded in the proteasomes (Blagosklonny, 1997; Haupt et al.,1997; Kubbutat et al., 1997). The p53 gene is located in chromosome 17p13.1. The coding protein is 53 kDa in size and consists of 393 amino acids residues (Nikolova et al., 2000). There are five major domains in p53 protein:

- The **N-terminal domain, (aa 1-42)** that is important for transcriptional activation as it has binding sites for gene expression inducing factors as TATA box binding protein (TBP) and TATA box binding protein-associated factors (TAFs) (Dutta et al., 1993; He et al., 1993; Li and Botchan, 1993; Lu and Levine, 1995; Thut et al., 1995; Unger et al., 1999).
- A **proline rich region (aa 63-97)** containing five repeats of PxxP sequence which interacts with-SH3- domains of proteins (Src tyrosine kinase). It is also playing a role in non-transcriptional activation of the intrinsic apoptotic pathway (Walker and Levine, 1996; Sakamuro et al., 1997; Venot et al., 1998).
- The **DNA-binding domain (aa 102-292)** is able to interact with specific DNA sequences of p53 target genes including *p21* and *mdm2*, to name a few (Bargonetti et al., 1993; Halazonetis et al., 1993; Pavletich et al., 1993; Wang et al., 1993; Ko and Prives, 1996). About 28% of mutations occur within the DNA-binding domain at

six hot spots of aa 175, 245, 248, 294, 273 and 282 (Raycroft et al., 1990; el-Deiry et al., 1993; Cho et al., 1994**;** Karen et al., 2002).

- The **C-terminal domain includes an oligomerization or tetramerization domain (a-a 323-356).** Moreover, it regulates the sub-cellular localization of p53 according to three nuclear localization signals (NLS). Therefore, a mutation in NLS1 (amino acids 316-325) causes p53 to locate in the cytoplasm, while NLS2 spanning from aa 369-375 and in NLS3 spanning from aa from 379-384) leads to location in cytoplasm and nucleus (Shaulsky et al., 1991).

- The **regulatory domain is spanning from aa 360-393** (Wang et al., 1993; Bakalkin et al., 1994; Bayle et al., 1995; Lee et al., 1995; Reed et al., 1995) (**Figure 7).**

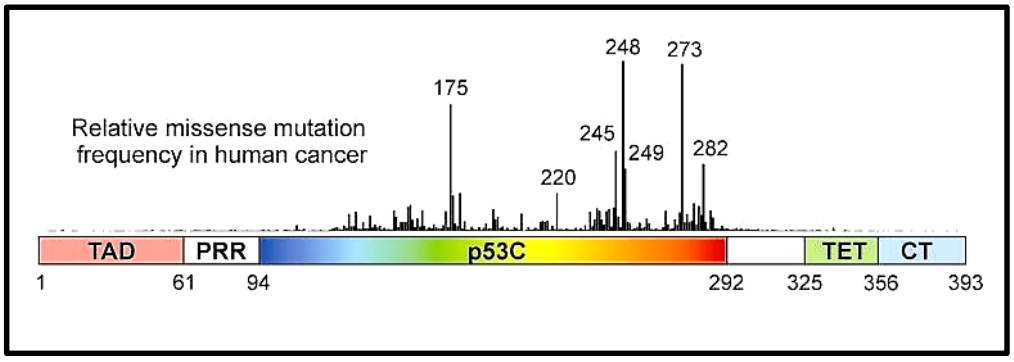

Figure 7: Domain structure of full-length p53.

The protein contains an N-terminal transactivation domain (TAD), followed by a proline-rich region (PRR), the central DNA-binding domain (p53C), the tetramerization domain (TET), and the C-terminus (CT). p53C, the domain where most cancer-associated p53 mutations are located, is highlighted with a rainbow color gradient from blue at its N-terminus to red at its C-terminus. The bars above the diagram indicate the relative frequency of oncogenic missense mutations at each residue according to version R11 (October 2006, N = 17,015) of the International Agency for Research on Cancer TP53 mutation database (**from Joerger and Fersht, 2008**).

p53 is a member of a small family of proteins involving two other members, i.e. p63 and p73 *(*Arrowsmith 1999; Levrero et al., 1999; Levrero et al., 2000) which show similar structural and functional features as p53 (Kaghad et al., 1997; Yang, Kaghad et al. 1998).

In response to genotoxic stresses, for example UV and cytotoxic drugs , oncogenic stress (Myc, Ras V12) and viral infection, p53 mediates DNA-damage response, via several kinases including Arf (alternate reading frame), ATM, ATR, chk1 and chk2 (protein kinases). Phosphorylation of p53 at Ser 20 and Ser 15 leads to its activation. This phosphorylation within or near the MDM2-binding domain results in stabilization of p53, preventing MDM2 from binding to p53 which consequently causes an accumulation of p53 in the nucleus (Giaccia and Kastan, 1998; Chehab et al., 1999). p53 is modified by acetyl transferases such as p300, CBP and PCAF that acetylate p53 at its C-terminal lysine residues (i.e Lys-370, Lys-371, Lys-372, Lys-381, Lys-382) leading in turn to activation of p53 DNA-binding activity (Gu and Roeder, 1997**;** reviewed in Kouzarides, 2000; Sterner and Berger, 2000) together with induction of transcriptional activity of numerous genes involved in cell cycle arrest (p21, 14-3-3α), DNA-repair (GADD45, Killer/Dr5), and apoptosis (BAX, PUMA, NOXA) or obstruct angiogenesis through up-regulation of thrombospondin. Taken together, these mechanisms reduce the risk of transformation of cells within a genetic and epigenetic lesion, preventing in turn tumour formation (Shieh et al., 1997; Unger et al., 1999; Chehab et al., 1999; Vousden and Lu, 2002; Yu and Zhang, 2005; Laptenko and Prives, 2006; Pelengaris and Khan, 2006; Rozan and El-Deiry, 2007) **(Figure 8).**

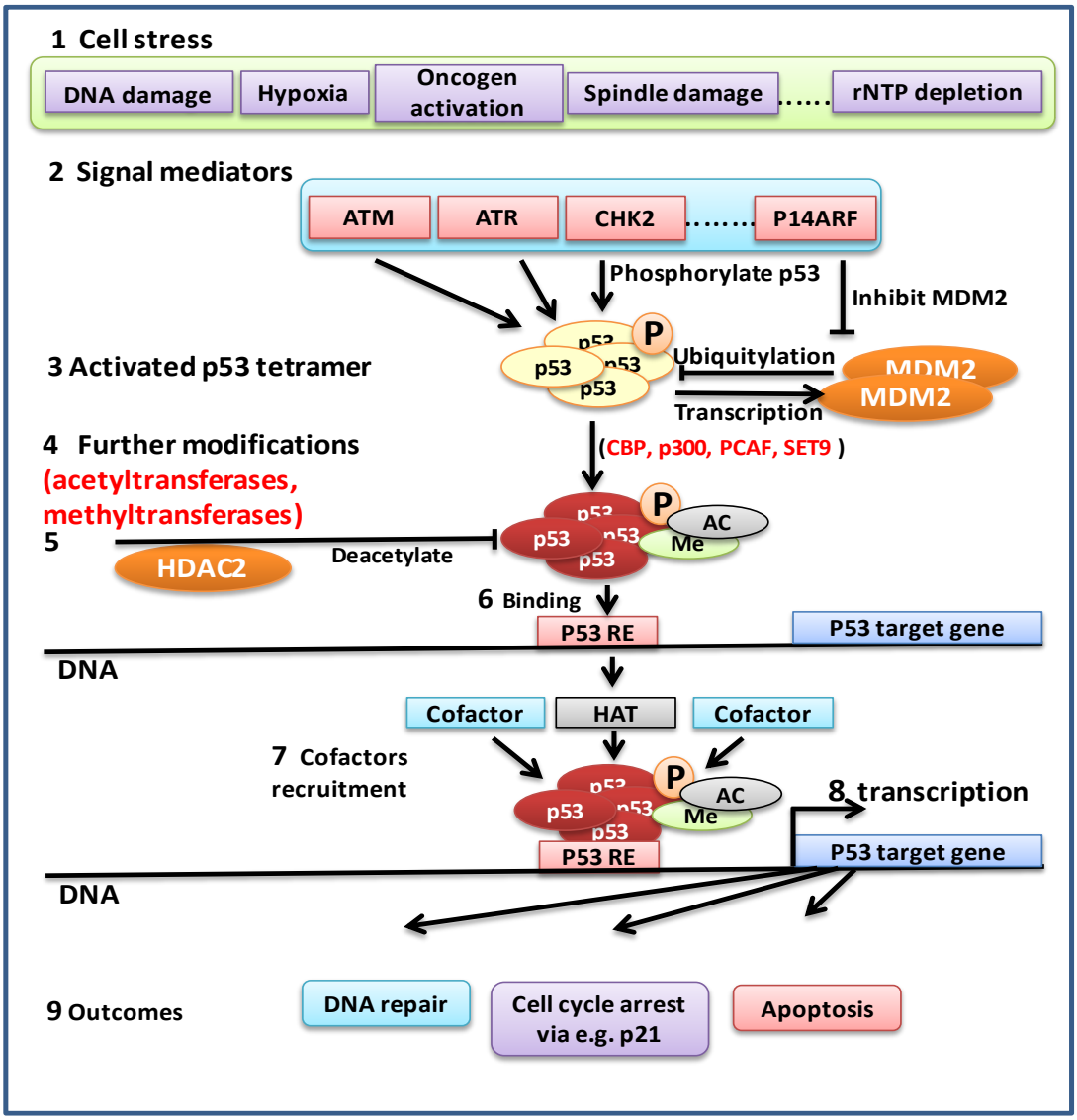

Figure 8: Activation and function of p53.

Step 1 and 2: In response to many types of cellular stress, signal mediator proteins are activated. ***Step 3:*** phosphorylation of p53 in its N-terminal domain preventing inturn its ubiquination by the action of MDM2 hence increase the p53 half-life which subsequently leads to p53 accumulation. ***Step 4:*** further p53 modifications takes place by acetyltransferases (CBP, p300, PCAF) and methyltransferases (SET9) which increase the p53 DNA-binding capacity. ***Step 5:*** deacetylation of p53 may takes place in responce to the deacetylase HDAC2 inhibiting inturn p53 binding to DNA. ***Step 6:*** binding of p53 tetramer to the p53 response element (RE). ***Step 7:*** recruitment of gene expression inducing cofactors such as histone acetyltransferases (HATs), TATA box-binding protein and TATA box-binding protein-associated factors (TAFs). ***Step 8:*** initiation of p53 target genes translation. ***Step 9:*** production of various proteins involved in DNA-repair, cell-cycle arrest, and apoptosis. ATM, ataxia telangiectasia mutated; CHK2, checkpoint kinase-2 **(modified from Riley et al., 2008).**

Malfunction of the p53 pathway has been reported as the most common hallmark for human tumours (Vogelstein et al., 2000; Vousden and Lane 2007). It has been documented that mutated and / or deleted p53 accounts for approximately 50% of all human cancers (Hollstein et al., 1991, Vogelstein et al., 2000; Petitjean et al., 2007). Mutations can be divided into **somatic cells mutations** as a result of DNA-damage and these mutations appear in the DNA-binding domain of p53. **Germline mutations** have been identified in Li-Fraumeni syndrome affected people, leading to a high risk for malignant tumour formation (Whibley et al., 2009).

1.4.2 $p76^{MDM2}$ and MDM4 interaction in the regulation of p53

In 1987, murine double minute 2 (*MDM2*) gene was found to be over-expressed in the spontaneously transformed 3T3-DM mouse cell line (Cahilly-Snyder et al., 1987). The human gene homolog (also called *MDM2* or *HDM2*) was found to be high in human sarcomas. More than 10% of 8000 human cancers, including lung or stomach cancer, showed elevation of the *MDM2* gene (Toledo and Wahl, 2006). Wild-type human and mouse *mdm2* gene encodes many mdm2 protein products. Some of these isoforms bind to p53.

The most frequent isoforms are two proteins with different molecular weights. One is the full length protein, $p85/90^{MDM2}$, another is the smaller splice variant $p74/76^{MDM2}$ in addition to $p54/57^{MDM2}$ (Olson et al., 1993; Haines et al., 1994; Gudas et al., 1995; Saucedo et al., 1999; Cheng and Cohen, 2007) **(Figure 9).**

In 1996, a structurally $p90^{MDM2}$ – homolog was identified (Shvarts et al., 1996, Marine et al., 2007). This protein was firstly named MDMX, and then it was called MDM4 (the human homolog is known as MDM4, MDMX, HDM4 or HDMX).

MDM4 was detected in 10-20% of more than 800 different tumours, including lung, colon, stomach and breast cancers (Toledo and Wahl, 2006). Strikingly, 65% of retinoblastomas show this regulator (Laurie et al., 2006). Moreover, high MDM4 levels were reported in approximately 65% of the melanomas examined (Gembarska et al., 2012).

Human $p90^{MDM2}$ and MDM4 are consisting of 491 and 490 amino acids respectively, and can be divided into three well identified domains **(Figure 10)**.

- N-terminal domain which is crucial for binding to Box I region of the p53-N-terminal domain leading to a disruption in p53 interaction with the transcriptional machinery as well as the transcriptional co-activator p300 (Joazeiro, and Weissman, 2000; Toledo and Wahl, 2007).

- Zinc-finger domain (Joazeiro, and Weissman, 2000; Toledo and Wahl, 2007).

- C-terminal RING domain that is characteristic to E3 ubiquitin ligases. It is also important for both homo- and hetero-dimerization, leading to more stability of MDM2 and MDM4 depending on their relative intracellular concentration (Toledo and Wahl, 2007; Joazeiro, and Weissman, 2000) **(Figure 10)**. The RING finger domain is crucial for mdm2 binding to RNA (Elenbaas et al., 1996). When mdm2 binds *p53* mRNA, it promotes translation of p53 protein (Naski et al., 2009; Gajjar et al., 2012) and impairs E3 ligase activity (Candeias et al., 2008).

$p76^{MDM2}$ isoform can not bind to p53 protein, as it lacks the N-terminal 49 amino acids of the full length protein $p90^{MDM2}$, which is the domain of $p90^{MDM2}$ involved in interaction with p53 (Chen et al., 1993; Olson et al., 1993; Haines et al., 1994; Saucedo et al., 1999). Hence, $p76^{MDM2}$ cannot promote p53 protein degradation via ubiquitination, but it can bind to *p53* mRNA and promote efficient translation of the mRNA (Naski et al., 2009). In contrast to other mdm2 family members $p76^{MDM2}$ acts positively towards p53 via antagonising the ability of $p90^{MDM2}$ to target p53 protein degradation via inhibiting $p90^{MDM2}$ association with p53 without affecting $p90^{MDM2}$ levels (Perry et al., 2000; Giglio et al., 2010).

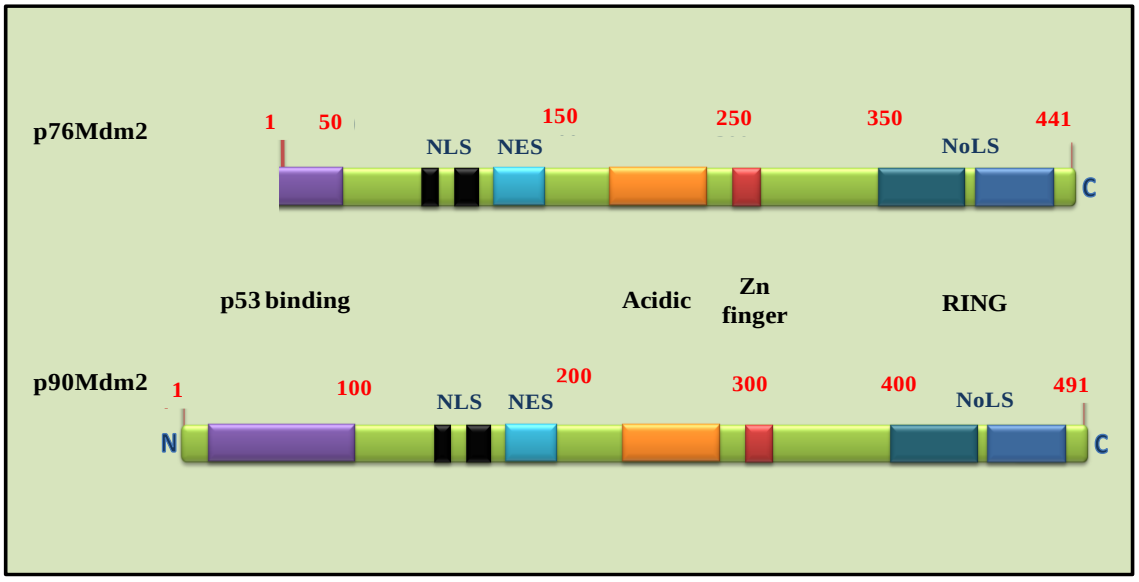

Figure 9: **Protein structural homology of p76^{MDM2} and p90^{MDM2}**.

p76^{MDM2} lacks the N-terminal 49 amino acids of the full length protein p90^{MDM2}, the fraction of p90^{MDM2} involved in interaction with p53 (Olson et al., 1993; Haines et al., 1994; Saucedo et al., 1999; Chen et al., 1993). Both have similar nuclear localization signal (NLS), nuclear export signal (NES), Zinc finger-(Zn), RING finger domain as well as nucleolar localization signal (NoLS).

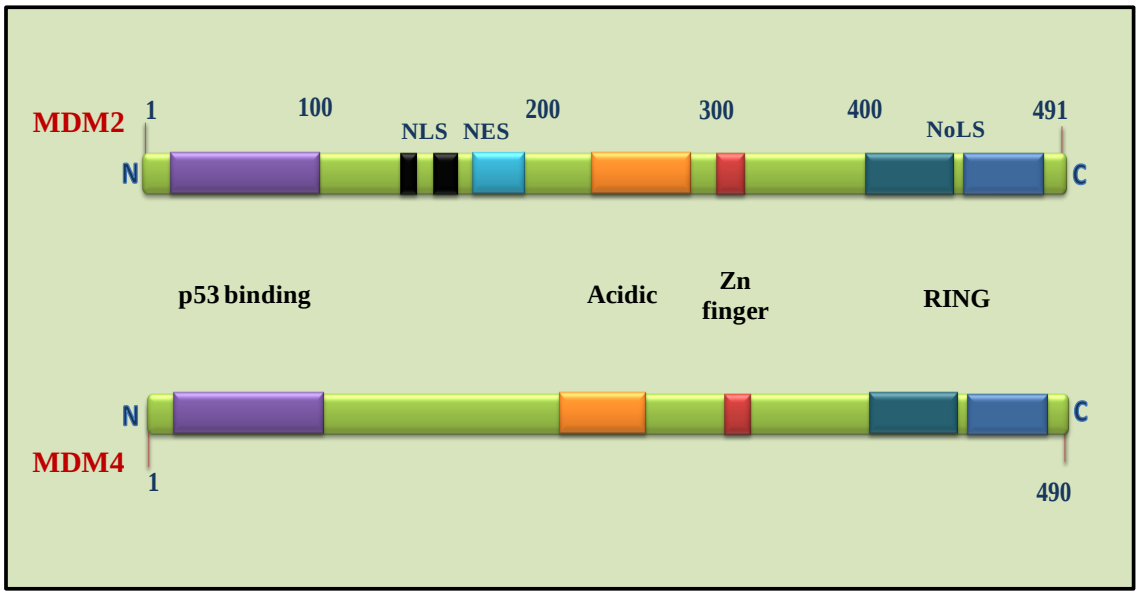

Figure 10: Protein structural homology of MDM2 and MDM4.

MDM2 and MDM4 have similar N-terminal p53 binding domain, Zinc finger-(Zn) as well as RING finger domain while Hdm2 characterizes by the presence of nuclear localization signal (NLS), nuclear export signal (NES) and nucleolar localization signal (NoLS).

p90^{MDM2}and MDM4 proteins control p53 levels and activity in two ways: independently and dependently.

- Independently p90^{MDM2} can function as an E3 ubiquitin ligase, catalysing not only the ubiquitination of p53, but also proteosomal degradation of itself as well as the action of MDM4 (Honda et al., 1997; Fang et al., 2000; Pan et al., 2003). p90^{MDM2} controls p53 activity in two distinct ways: 1. via binding to the N-terminal domain of p53, preventing it from binding gene expression inducing factors causing in turn a blockade of the p53-dependent transcriptional machinery. 2. by ubiquitinating p53 and labelling it for proteasomal degradation (Oliner et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997; Honda and Yasuda, 2000). Importantly, p90^{MDM2} / p53 binding alone in the absence of Mdm2 E3 ubiquitin ligase activity is insufficient to suppress p53 activity (Itahana et al., 2007). Here it s of note that p90^{MDM2} can also promote mono-ubiquitination which does not directly cause p53 degradation. It can promote the export of p53 from the nucleus to the cytoplasm especially to the mitochondria. p90^{MDM2} can also promote other modifications of p53 (Li et al., 2003; Carter et al., 2007).

  Similar to p90^{MDM2}, MDM4 can bind to p53 via its p53 binding domain which is located on its N-terminus disturbing transcriptional activity of p53. Therefore MDM4 has been hypothesized as a negative regulator of p53, but without triggering direct degradation of p53, as it does not have ubiquitin ligase activity (Honda et al., 1997; Fang et al., 2000; Pan and Chen., 2003; Danovi et al., 2004). However, some studies have reported a p53-positive regulation by MDM4, as they claimed its ability to inhibit p90^{MDM2}- mediated p53 degradation by

competing with $p90^{MDM2}$ for p53-binding, resulting in turn in accumulation of p53 (Sharp et al., 1999; Jackson and Berberich, 2000; Stad et al., 2000).

- Despite of the independent MDM2 and MDM4 effect on p53, other studies highlighted the possibility that both proteins work together in control of p53 levels / activity as $p90^{MDM2}$ alone is a relatively ineffective E3 ubiquitin ligase (Kawai et al., 2007). It becomes more efficient for ubiquitinating p53 after heterodimerization with MDM4 (Linares et al., 2003). MDM4 dimerizes with $p90^{MDM2}$, forming MDM protein complex which binds to p53, leading then to its inactivation. Both, MDM proteins co-localize with p53 at its DNA-consensus sequences, supporting the predominance of this complex in the nucleus (Tang et al., 2008; Wade and Wahl, 2009). Both, MDM2 and MDM4 proteins, play an essential role in controlling each other as MDM4 stabilizes $p90^{MDM2}$ by interfering with its auto-ubiquitination. However, MDM4 has also been reported being ubiquitinated and degraded by $p90^{MDM2}$ (Pan and Chen, 2003). The ratio of $p90^{MDM2}$ / MDM4 can be used to explain the $p90^{MDM2}$ status in different situations i. e. $p90^{MDM2}$ forms homodimers and is degraded by itself through ubiquitination, if the ratio is high. On the contrary, $p90^{MDM2}$ is stabilized, if the ratio is low (Linke et al., 2008).

p53 binds to p53-responsive elements, located within the $p90^{MDM2}$ gene, and promote its transcription, thereby seting up a negative feedback regulatory loop (Barak et al., 1993; Perry et al., 1993), while p53 cannot transactivate MDM4. Because of this scenario, protein levels of $p90^{MDM2}$ fluctuate widely upon p53 activation, whereas MDM4 level remain relatively constant (Barak et al., 1993; Perry et al., 1993).

Alterations in expression, localization as well as in activity of MDM proteins has been reported under certain stress and pathological conditions. In response to UVR MDM4 is phosphorylated by CHK1 which increases its association ability to 14-3-3γ, leading in turn to its retention in cytoplasm, promoting activation of p53 transcriptional function and G1 arrest. This p53 activation is accompanied by an increase in its stabilization and decrease in ubiquitination, suggesting that cytoplasmic phosphorylated MDM4 (MDM4S367P) inhibits $p90^{MDM2}$ ubiquitin ligase function (Jin et al, 2006). In 2008 it was suggested that under stress conditions endogenous MDM4 stabilizes induced p53 due to its antagonism towards MDM2 (Barboza et al., 2008) **(Figure 11)**.

In response to DNA-damage, the ATM-pathway is activated. ATM is phosphorylating MDM4 either directly or indirectly through Chk2 kinase. This phosphorylation of MDM4 enhances its degradation by MDM2. One consequence is activation of p53 (Chen et al., 2005b; Pereg et al., 2005; Pereg et al., 2006a,b) **(Figure 11)**.

In 2009 Salem and colleagues reported, that epidermal $p76^{MDM2}$ levels were significantly increased in patients with vitiligo in association with enhanced p53 activity. Based on these results, these authors suggested a crucial role of $p76^{MDM2}$ in controlling DNA-damage / repair and prevention of photodamage and non-melanoma skin cancer in vitiligo (Salem et al., 2009).

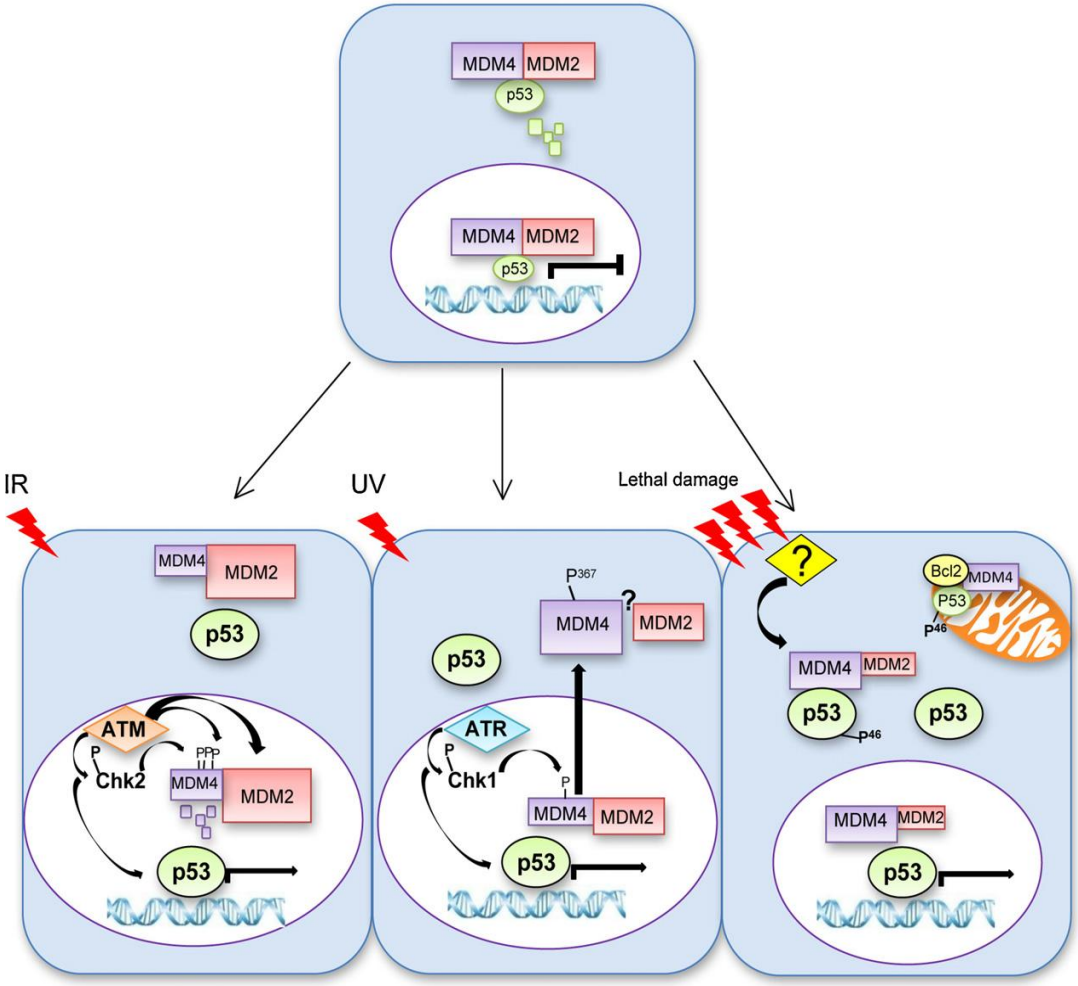

Figure 11: Modes of MDM4 function following different DNA damage.

Upon IR exposure, ATM and its target Chk2 phosphorylate MDM4 followed by rapid degradation by MDM2, resulting in p53 activation. Upon UV exposure, Chk1 phosphorylates MDM4 and mediates its nuclear export, resulting in p53 activation. After lethal DNA-damage (e.g. by high UV doses, adramycin and cisplatin), MDM4 promotes p53 phosphorylation at S46 and facilitates its recruitment to mitochondria, binding to Bcl2, and triggering of the mitochondrial apoptotic pathway. IR (infra red); ATM (ataxia telangiectasia mutated); CHK1 (checkpoint kinase-1); CHK2 (checkpoint kinase-2); ATR (ataxia telangiectasia and Rad3-related protein); Bcl-2 (B-cell lymphoma-2) (**from Mancini et al., 2010).**

1.4.3 SPARC (Secreted protein acidic and rich in cysteine)

SPARC (also known as osteonectin or basement-membrane-40, BM-40), is a non-structural glycoprotein component of extracellular matrix which was firstly identified in 1981 by Termine and colleagues as osteonectin, a bone-specific phosphoprotein that can bind to collagen fibrils and hydroxyapatite (Termine et al., 1981; Tai and Tang, 2008). Later SPARC was known as a modulator for cell-matrix interactions, particularly during normal tissue development, remodelling and repair as well as neoplastic transformation as it functions as a regulator of the deposition of extracellular matrix (Bradshaw and Sage, 2001; Brekken and Sage, 2001; Chlenski and Cohn, 2010).

SPARC is a 32.5 kDa protein consisting of three different domains **(Figure 12)**;

- Domain I: N-terminal domain which is an acidic region rich in asparagine (Asp) and glutamate (Glu), which can bind to 5–8 calcium ions, through the helix-turn-helix structural domain (EF-hands motifs) (Hohenester et al., 1997, Ribeiro et al., 2014).
- Domain II: Follistatin (FS)-like domain which is a cysteine-rich region. This domain encloses bioactive peptides that have stimulatory as well as inhibitory effects on endothelial cells (Hohenester et al., 1997; Ribeiro et al., 2014). Bioactive peptides that show inhibitory effects on endothelial cells, involve peptide 2.1 which is structurally similar to epidermal growth factor-like S hairpin and inhibits endothelial cell proliferation. Peptide FS-E is reported as an inhibitor of *in vitro* migration as well as *in vivo* angiogenesis of endothelial cell (Hohenester et al., 1997; Chlenski et al., 2004; Ribeiro et al., 2014), Peptide FS-K inhibits proliferation of endothelial cells (Ribeiro et al., 2014), while peptide

2.3 enhances proliferation and angiogenesis of these cells (Lane and Sage, 1994; Hohenester et al., 1997; Ribeiro et al., 2014).

- Domain III: Extracellular Ca^{2+} domain binding to extracellular Ca^{2+} ions through EF-hands motifs. This domain encloses the peptide 4.2, which supports migration of endothelial cells, but is inhibiting proliferation (Hohenester et al., 1997; Kupprion et al., 1998; Ribeiro et al., 2014). SPARC can bind to different types of collagen including fibril-forming collagen types I, III, and V, as well as the basement membrane collagen type (IV) in a Ca^{2+} dependent manner (Termine et al., 1981., Hohenester et al., 1997; Ribeiro et al., 2014).

Receptors for SPARC are not detected yet, while some studies suggested that SPARC may compete with other ligands for interacting with their receptors (Bradshaw et al., 2001).

SPARC plays a fundamental role in promoting metastasis via acting with other extracellular matrix components. It functions as a de-adhesive molecule and as a cell cycle inhibitor *(*Yan and Sage, 1999; Bradshaw and Sage, 2001; Brekken and Sage, 2001; Chlenski and Cohn, 2010). Many cell types including platelets, osteoblasts, endothelial cells and fibroblasts are SPARC-secreting cells (Termine et al., 1981; Brekken and Sage, 2000; Alford and Hankenson, 2006).

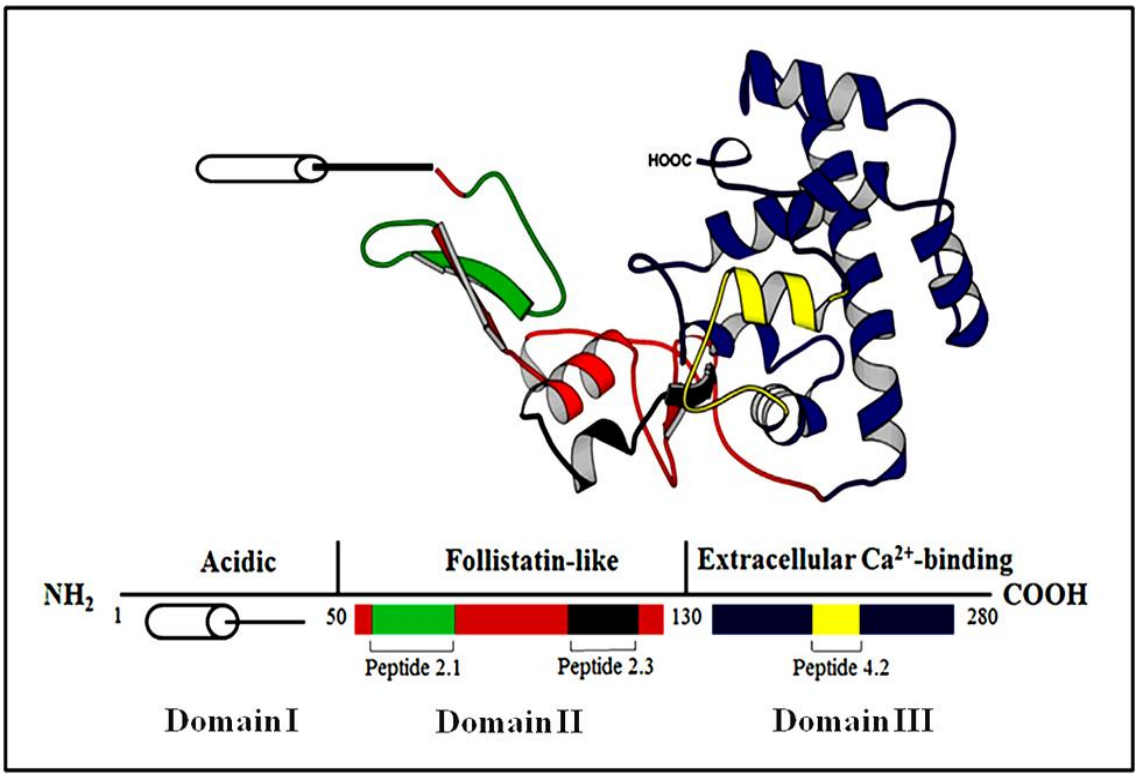

Figure 12: Structure of human SPARC protein.

A ribbon diagram derived from crystallographic data shows the three domains of SPARC. The follistatin-like domain, shown in red (except the peptide 2.1), aa 55–74, and the peptide 2.3 (aa 114–130) shown in green and black, respectively. Domain III is shown in blue (except the aa 255–274, peptide 4.2 shown in yellow) **(adapted from Hohenester et al., 1997).**

SPARC exhibits different patterns of expression and activity according to the tumour position as well as its surrounding stromal cells (Tai and Tang, 2008).

In 2011, it was reported that depletion of SPARC in melanoma cells results in an activation of p53 and induction of p21, leading in turn to G2/M cell cycle arrest and tumour growth inhibition (Fenouille et al., 2011a;b).

Expression of SPARC was found to be regulated by several factors including some members of the transforming growth factor beta (TFG-β) super-family including TGF-β1, activin and bone morphogenetic protein (BMP). All increase the expression of either SPARC protein or its mRNA (Nakamura et al., 1996; Damjanovski et al., 1998). Moreover, VEGF induces the expression of SPARC in human vascular endothelial cells (Kato et al., 2001; Weninger, et al., 1996) but this induction of SPARC might be a negative regulatory feedback mechanism as it was reported that SPARC binds VEGF and prevents activation of VEGFR1 (Kupprion et al., 1998; Nozaki et al., 2006). In this context it is of interest that VEGF was detected in keratinocytes in the skin and this expression was established to be enhanced by NO and H_2O_2 (Brauchle, et al., 1996; Frank et al., 1999).

SPARC regulates also the activity of basic fibroblast growth factor (bFGF), (Francki et al., 2004; Hasselaar and Sage, 1992) by inhibiting bFGF-stimulated migration of endothelial cells (Hasselaar and Sage, 1992). As SPARC induces the expression and secretion of TGF- β1 *in vitro* and *in vivo,* it is tempting to suggest that there is a reciprocal regulatory feedback loop between SPARC and TGF-β1 (Bassuk et al., 2000). Moreover, TGF- β1 is known as a negative regulator for melanogenesis as melanocytes, established under *in vitro* conditions in the presence of TGF- β, show a decrease of fully mature melanosomes and inhibition of melanin formation in association with clinical hypopigmentation (Martínez-Esparza et al., 2001).

1.4.4 Transforming growth factor- β1 (TGF- β1)

TGF- β1 is a secreted multifunctional growth factor, expressed as a 55 KDa polypeptide. It dimerises, forming a precursor molecule that cleaves in the Golgi apparatus to produce its small latent form. Latent TGF-β binding protein (LTBP) supports latent TGF-β release to the extracellular matrix and its activation (Hyytiainen et al., 2004).

TGF- β1 plays an important role in regulation of many biological processes, involved in tissue homeostasis, including cell proliferation and differentiation, apoptosis, deposition of extracellular matrix and cell adhesion (Derynck and Feng, 1997; Massagué, 1998; Roberts, 1998; Gumienny & Padgett, 2002; Lutz & Knaus, 2002). Moreover, TGF- β1 was reported as a negative regulator for melanogenesis, causing degradation or inactivation of tyrosinase. It down-regulates the production of MITF, TRP-1 and TRP-2, decreasing in turn the percentage of fully mature melanosomes, consequently leading to inhibition of melanin biosynthesis in association with clinical hypopigmentation (Martínez-Esparza et al., 1997; Martínez-Esparza et al., 2001; Kim et al., 2003).

Effects of TGF-β1 take place via its binding to its transmembrane receptor serine / threonine kinases complex, TGF-βRI and TGF-βRII leading to recruitment and phosphorylation of Smad2 and / or Smad3 which in turn heteromerize with Smad4 and translocate into the nucleus, where it regulates the transcription of different genes including p21 (Datto et al, 1995; Li et al., 1995) **(Figure 13).** Kim and colleagues, proposed in 2005 that TGF-β1-induced receptor activation stimulates not only Smad pathway, but also a parallel H_2O_2-mediated ERK pathway, resulting then in p21 up-regulation (Kim et al., 2006). Growth suppression and apoptosis inhibition properties of TGF- β1 involve induction of p21 expression through p53-independent mechanism and reduction of c-myc, CDC25A and Id family members **(Figure 13).** However, the data

are controversial. While some studies reported that malignant melanoma cells appear to be resistant to the TGF-β1-mediated effect (Rodeck et al., 1994; Krasagakis et al., 1999; Rodeck et al., 1999; Hoek et al., 2006), others claimed TGF-β1as a pro-oncogenic factor (Javelaud et al., 2008).

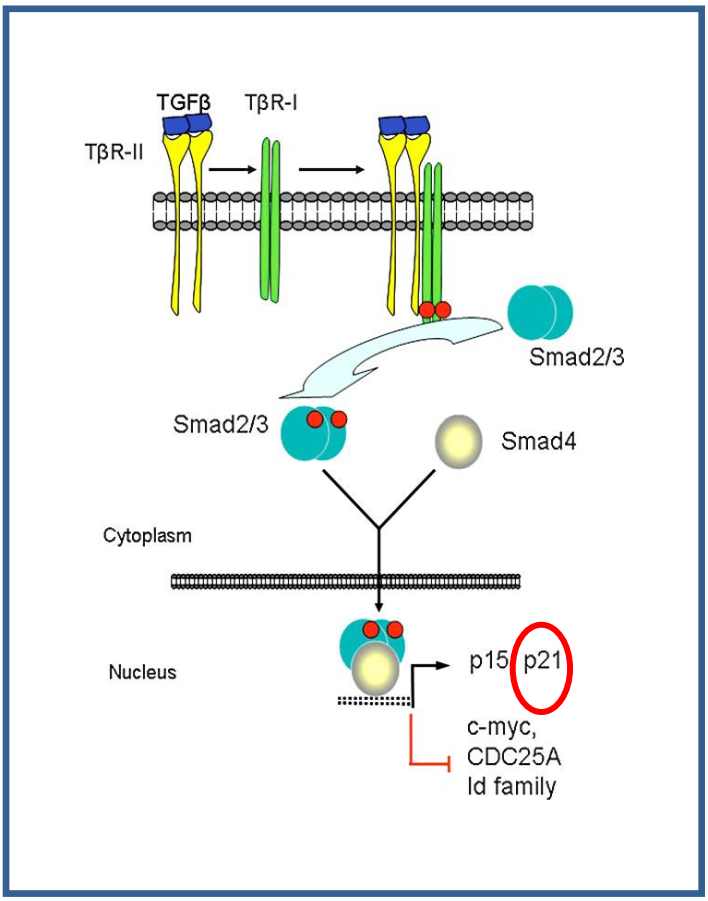

Figure 13: p21 expression through p53-independent mechanism via TGFβ-1 canonical signal transduction pathway.

At the cell surface, TGFβ-1 assembles a complex of transmembrane receptor serine / threonine kinases (types I and II) and induces trans-phosphorylation and activation of the type I receptor (TβR-I, ALK5) by the type II receptor kinase (TβR-II). Activated TβR-I phosphorylates the main TGF-β downstream effectors, Smad2 and Smad3, at C-terminal serines. Activated Smad2 and Smad3 then associate with Smad4 and the complexes translocate into the nucleus and regulate transcription of target genes, involved in TGFβ-induced growth inhibition. Downregulation of c-myc, CDC25A and Id family members and up-regulation of p15 and p21 CDK inhibitors are key events in this response **(modified from Lasfar and Cohen-Solal; 2010).**

2. Aim of the study (Part I)

Surprisingly patients with vitiligo have no increased risk for development of solar induced non melanoma skin cancer (i.e. basal cell carcinoma, squamous cell carcinoma and actinic keratoses) despite massive accumulation of H_2O_2 and $ONOO^-$ in association with a seriously perturbed antioxidant defence system and the lack of pigment (Calanchini-Postizzi and Frenk, 1987; Schallreuter et al., 1991b; Schallreuter et al., 1999b; Schallreuter et al., 2002; for review Schallreuter et al., 2008b; Salem et al., 2009; Teulings et al., 2013, Schallreuter, 2014).

As patients with vitiligo have a persistent up-regulated wild type functioning p53 together with increased p21 throughout the entire epidermis in association with an efficient DNA-repair, it has been suggested that this cascade could be valid in prevention of solar induced photo-damage and solar induced skin cancer in these patients (Schallreuter et al., 2003; Salem et al., 2009). As a consequence it was suggested that this p53 accumulation is associated with a perturbed degradation by the action of high $p76^{MDM2}$ levels. This protein antagonises binding of p53 to its regulator $p90^{MDM2}$ which is responsible for ubiquitination of p53 (Schallreuter et al., 2003; Salem et al., 2009). In this context it was shown that another MDM2 family member, i.e. MDM4 togther with its regulator MDM4phospso, can control p53 levels and activity (Honda et al., 1997; Sharp et al., 1999; Fang et al., 2000; Jackson and Berberich, 2000; Stad et al., 2000; Pan and Chen, 2003; Danovi et al., 2004). Moreover, SPARC was recently reported as a part of cellular anti-stress behaviour via inactivating the p53 / p21 pathway through AKT-mediated MDM2 phosphorylation (Fenouille et al., 2011a;b).

Therefore the first aim of this thesis was to get a better understanding of the mechanism behind the constantly up-regulated p53 together with increased p21 in vitiligo. It appeared tempting to follow those proteins involved in negative and / or positive

regulation of p53 and p21, including $p76^{MDM2}$, MDM4 and SPARC. As TGF-β1 can control p21 expression (Kim et al., 2006), we included this protein in our study. In this context it became of interest that VEGF-A induces expression of SPARC (Kato et al., 2000; Weninger, et al., 1996). Therefore it seemed a logical consequence to include this arm for SPARC regulation.

3. Materials and methods

3.1 Cell culture

3.1.1 Establishment and maintenance of cell cultures

Primary epidermal melanocyte cultures, established from waste skin after aesthetic surgery, were grown in T75 cm^2 cell culture flasks. Briefly, fat from the skin was removed, followed by a quick washing step in phosphate buffered saline (PBS). Incisions were made on the epidermis to ease separation of the dermis from the epidermis and the skin samples were then incubated with dispase (Roche Applied Science, Roche Diagnostics Corporation, Indianapolis, USA) for 12 hours at 4°C. The following day the epidermis was peeled and washed in 5% penicillin / streptomycin (P/S) (Life Technologies, Paisley, UK) and 5% fungisone (Life Technologies, Paisley, UK) and placed into sterile PBS. To produce a cell suspension from the processed epidermis, 1x trypsin / EDTA (TE) (Life Technologies, Paisley, UK) was added and incubated for 10 minutes at 37^oC. The reaction was stopped by the addition of medium 154, containing keratinocyte specific growth factors, 0.1mM calcium chloride (Cascade Biologics, Mansfield, Nottinghamshire, UK) and 1% P/S. The suspension was centrifuged at 500*g* for 10 minutes. The supernatant was removed and the cell pellet, containing both melanocytes and keratinocytes, was resuspended in the prepared medium and seeded into 75cm^2 culture flasks (Scientific Laboratory Supplies, Nottingham, UK). The medium was changed every 2 days. In order to obtain single cell populations, separation of melanocytes from keratinocytes was required. This was achieved via selective trypsination, whereby melanocytes detach from the flask in approximately 1-2 minutes, when TE solution is added, whereas keratinocytes take

around 5 minutes or more. Once detached, melanocytes were transferred to complete 2:1 MEM medium. MEM medium was from Invitrogen, Paisley, UK, containing, penicillin (100 units/ml) and streptomycin (100µg/ml), L-glutamine 2mM, fungizone 0.2%, fetal calf serum 2%, concentrated non-essential amino acid mixture 1%, endothelin-1 (5ng/ml), basic fibroblast growth factor (5ng/ml) and Ciprofloxacin 0.4%) in 500ml. One fold of keratinocyte serum free medium from Invitrogen, Paisley, UK was added. 500 ml medium contained bovin pitutary extract (25 mg/ml), epidermal growth factor (2.5µg/ml), fungizone 0.2%, penicillin (100 units/ml) and streptomycin (100 µg/ml) 5X, L-glutamine 2 mM, ciprofloxacin (0.4%). Cells were fed every 48-72 hours. Melanoma cell cultures (FM 55 cells and FM 94 cells) were passaged into T75 cm^2 cell culture flasks and fed every 48 hours with complete RPMI 1640 medium (500ml RPMI 1640 (Invitrogen, Paisley, UK) containing penicillin (100 units/ml) and streptomycin (100µg/ml), L-glutamine 2mM, fungizone 0.2%, fetal calf serum (10%), ciprofloxacin (0.4%). Briefly, cell culture flasks were removed from the incubator and the medium was discarded into 2% Virkon. Subsequently, 10 ml of fresh medium was added to the cell culture flask under sterile conditions and the flask was placed back into the incubator.

3.1.2 Thawing of frozen cell cultures

Stored frozen cells were removed from liquid nitrogen and immediately placed in to a water bath at $37^{O}C$. Before thawing of the cells, 10 ml of complete medium was added to each T75 cm^2 cell culture flask. The contents of the cryovials were then added gradually to the medium and the flasks were placed in the incubator for at least 24 hours to allow cells to attach to the surface of the flasks.

3.1.3 Passaging of cell cultures

Cells were passaged, once the culture was 80% confluent and actively proliferating. The medium was removed from the T75 cm^2 cell culture flasks and discarded into 2% Virkon. The cells were then rinsed with approximately 8 ml of PBS. Once PBS had been discarded into 2% Virkon, 1 ml of trypsin/EDTA was added to each flask covering the entire cells and then incubated at 37^0C for 2-3 minutes until cells started to detach from the bottom of the flasks. Detachment of the cells from the culture flasks was observed under an inverted microscope. After complete detachment of the cells, 30 ml of complete medium were added to each flask to inactivate the trypsin / EDTA action. The total content of each flask was then split into three flasks, and returned to the incubator for further growth.

3.1.4 Freezing of cell cultures

Cells were trypsinized and pelleted by centrifugation at 1000rpm for 3 minutes. The cell pellet was resuspended in freezing down solution (dimethyl sulphoxide (DMSO) 10%, fetal calf Serum (90%) and transferred to 1ml cryovials. The cryovials were placed in isopropanol bath for 24 hours at -80 ^{O}C in order to prevent cell damage. Thereafter cells were transferred to liquid nitrogen for long term storage.

3.2 Immunocytochemical Methods

3.2.1 Cell culture chamber slide preparation

To investigate the expression of different proteins in melanocytes by *in vitro* immuno-fluorescence, melanocytes (100 µl cell suspension) were initially transferred to each well of chamber slides (Nalge Nunc International, Naperville, IL, USA) followed by the addition of 300 µl medium to each well. Once the culture was approximately 70-80%

confluent, the medium was removed and discarded into 2% Virkon. Cells were washed 3-4 times with sterile 1x PBS, pH 7.4, followed by fixation in cold methanol at -20°C for 10 minutes. Slides were stored at -20°C until future work.

3.2.2 *In vitro* immuno-fluorescence labelling

Frozen slides were allowed to defrost at room temperature (RT) for about 10 minutes, followed by dehydration in 1x PBS for 5 minutes. Fixation was performed by immersing slides for 6 minutes in cold methanol (-20°C). The slides were then washed for 5 minutes in 1x PBS, followed by blocking with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) for 90 minutes at RT. Slides were washed in 1x PBS for 5 minutes, followed by overnight incubation at 4°C with the primary antibodies, diluted in 1% NDS. Thereafter, slides were washed twice in 1x PBS for 5 minutes, followed by another wash once in Tween-20 (0.05%) for 5 minutes and then once again in 1x PBS for 5 minutes, incubated for 1 hour at RT with a fluorescent secondary antibody in a dilution of 1:50 in 1% NDS for 1 hour (donkey anti-rabbit, rat, mouse or sheep, depending on the primary antibody that was used (Jackson Immuno Research Laboratories, Cambridgeshire, UK). Slides were washed twice in 1x PBS for 5 minutes, then once in Tween-20 for 5 minutes, followed by a final wash in 1x PBS for 5 minutes. Finally, slides were dried and mounted, using Vectashield Mounting Medium containing 4, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and covered with a coverslip. Viewing of the pictures was carried out by using a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture programme (Nikon, Europe). Control staining was carried out

by omitting the primary antibody from the staining procedure and substitution of 1% NDS / PBS. Antibodies used in our experiments are listed in (**Table 1).**

Antibody	Dilution	Incubation time/T	Source	Company	Cat. No.
Alexa 488	1:50	1 hour/RT	Donkey anti-Rabbit	Invitrogen, Paisley, UK	A21206
Alexa 594	1:50	1 hour/RT	Donkey anti-mouse	Invitrogen, Paisley, UK	A21203
catalase	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16731
MDMX	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16058
NKI/beteb	1:50	Overnight/ 4°C	Mouse monoclonal antibody	Caltag-Medsystems Ltd	MON7006-1
SPARC	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab14174
TGF-β1	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab66043
VEGF-A	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab46154

Table 1

List of primary and secondary antibodies and conditions for *in vitro* immuno-fluorescence labelling in melanocytes and melanoma cells.

3.3 Immunohistochemical Methods

3.3.1 Cryosection preparation

A 3 mm punch biopsy of full thickness skin was taken from consented normal healthy controls (n=10) and patients with vitiligo (n=20) under local anaesthesia from the volar proximal arm. The tissue samples originated from Professor Schallreuter`s tissue library (collected during 1992-2004). All skin biopsies were embedded in optimal cutting temperature compound (OCT) (Sakura, RA Lamb, Eastbourne, UK) and placed for about 5 minutes in liquid N2 to allow the skin to be shock frozen. 3-5 μm sections of this skin were cut using a Leica CM3050 S cryostat (Leica Microsystems, Milton Keynes, UK) and placed onto the poly-L-lysine coated slides (Sigma, Pool, Dorset, UK). Slides were saved at -80°C for future work.

3.3.2 *In situ* immuno-fluorescence labelling

Frozen slides were allowed to defrost at RT for 10 minutes followed by dehydration in 1x PBS for 5 minutes. Fixation was performed by immersing slides for 10 minutes in ice cold-methanol. Then slides were washed in 1x PBS for 5 minutes, followed by blocking with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) for 90 minutes at RT. Slides were washed once in 1x PBS for 5 minutes, followed by another wash once in Tween-20 (0.05%) for 5 minutes and then twice again in 1x PBS for 5 minutes each. Afterward slides were incubated overnight at 4°C with the primary antibodies, diluted in 1% NDS. Thereafter, slides were washed once in 1x PBS for 5 minutes, followed by another wash once in Tween-20 (0.05%) for 5 minutes and then twice again in 1x PBS for 5 minutes each. Then, slides were

incubated for 1 hour at RT with a fluorescent secondary antibody in a dilution of 1:50 in 1% NDS for 1 hour (donkey anti-rabbit or mouse, depending on the primary antibody that was used) (Jackson Immuno Research Laboratories, Cambridgeshire, UK). Slides were washed once in 1x PBS for 5 minutes, then once in Tween-20 for 5 minutes followed by two washes in 1x PBS for 5 minutes each. Finally, slides were dried and mounted using Vectashield Mounting Medium containing 4, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and covered with a coverslip. For double immuno-fluorescence, after the incubation of tissue sections with the first secondary antibody they were washed in PBS and instead of being mounted and coverslipped, blocking with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) was carried out. The whole procedure was repeated using the second primary and secondary antibodies. The first secondary antibody was FITC-labelled and the second secondary antibody was TRITC-labelled. Negative control staining was carried out by omitting the primary antibody from the staining procedure and substitution of 1% NDS / PBS. Viewing the pictures was carried out by using a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture program (Nikon, Europe). The antibodies and dilutions used are listed in (**Table 2).**

Antibody	Dilution	Incubation time/T	Source	Company	Cat. No.
Alexa 488	1:50	1 hour/RT	Donkey anti-Rabbit	Invitrogen, Paisley, UK	A21206
Alexa 594	1:50	1 hour/RT	Donkey anti-mouse	Invitrogen, Paisley, UK	A21203
catalase	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16731
MDM2	1:20	Overnight/ 4°C	Mouse monoclonal antibody	Santa cruz biotechnology	sc-965
MDMX	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16058
MDMX-phospho	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab70236
5-nitro-tyrosine	1:50	Overnight/ 4°C	Mouse monoclonal antibody	Abcam, Cambridge, UK	ab78163
NKI/beteb	1:50	Overnight/ 4°C	Mouse monoclonal antibody	Caltag-Medsystems Ltd	MON7006-1
p21	1:5	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab7960
p53	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab1431
SPARC	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab14174
TGF-β1	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab66043
VEGF	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab46154

Table 2: List of primary and secondary antibodies and conditions for *in situ* immuno-fluorescence labelling.

3.3.3 Quantification of fluorescence intensity

To quantify the fluorescence, ImageJ version 1.37 was utilised (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). The region to be quantified was highlighted and the mean intensity in relation to the area was obtained. This was repeated for the rest of the layers. The procedure was repeated in at least three epidermal sections of each patient and the mean value was obtained and plotted using Microsoft Excel (Microsoft Corporation, USA). Significance of data was analysed statistically using the two-tailed unpaired t-test.

3.4 SDS-PAGE and Western Blot

3.4.1 Protein extraction from cells

Media of melanocytes cell cultures was removed from the T75 cm^2 cell culture flasks and discarded into 2% Virkon. Cells were washed with PBS. Trypsin/EDTA was added slowly to each flask. The flasks were then incubated at 37^0C for 2-3 minutes until cells started to detach when 10ml of complete medium was added to each flask for inactivation of the trypsin/EDTA. The cell suspension was transferred to 25 ml universal tube. Additional 10ml medium was added to the flask to wash out any residual cells. Cells were then pelleted by centrifugation at 1200rpm for 6 minutes. The cell pellets were then resuspended in sample buffer (2.5 ml glycerol, 0.185 g EDTA, 1.5 g sodium dodecyl sulphate (SDS), 0.19 g tris, 25 ml distilled water) according to the volume of pellet. Cell suspensions were then transferred to 1.5 ml Eppendorf tubes. Protease inhibitor cocktail (1:20) was added and mixed well. Eppendorf tubes were placed in ice for three hours and were vortexed from time to time (approximately 30 minutes). Eppendorf tubes were then centrifuged at 13000 rpm for 5minutes at 4^0C. The supernatant was placed in -20^0C freezer until use.

3.4.2 Protein extraction from tissue

3 mm punch biopsies of full thickness skin plus 200µl of sample buffer (2.5 ml glycerol, 0.185 g EDTA, 1.5 g sodium dodecyl sulphate (SDS), 0.19 g tris, 25 ml distilled Water) were grinded mechanically using mortar and pestle. Tissue suspensions were then transferred to 1.5 ml Eppendorf tubes. Protease inhibitor cocktail (1:20) was added and mixed well. Eppendorf tubes were placed in ice for 30 minutes and vortexed every 10 minutes. Eppendorf tubes were then centrifuged at 13000 rpm for 5 minutes at 4^0C. The supernatant was placed in -20^0C freezer until further use.

3.4.3 Quantification of protein content in cells and tissue extracts (Bradford protein assay)

Five µl of protein standards (listed below) or protein samples of interest were added to the appropriate well on a 96 well-plate in triplicate following the manufacturer`s protocol. Briefly, to each well, 25 µl of reagent A' and 200 µl of reagent B were added. The plate was agitated for 20 minutes using a plate shaker before being placed in a plate reader. The absorbance of the samples was measured in each well at 750 nm. After calculating protein concentration in each cell extract protein aliquots were taken for loading in SDS-PAGE.

Bovine Serum Albumin (BSA) Stock Sample Buffer 10 ml / Bovine Serum Albumin 0.1 g (final concentration of 10 mg/ml)

Protein Standards (Bovine Serum Albumin)

Blank: 1000 µl sample buffer, 200 µl/ml: 980 µl sample buffer + 20 µl BSA stock
400 µl/ml: 960 µl sample buffer + 40 µl BSA stock, 600 µl/ml: 940 µl sample buffer + 60 µl BSA stock, 800 µl/ml: 920 µl sample buffer + 80 µl BSA stock, 1000 µl/ml: 900 µl sample buffer + 100 µl BSA stock

3.4.4 SDS-PAGE of protein samples

Electrophoresis analysis of protein samples was implemented by preparing 10% resolving gel [2 ml 30% acrylamide, 1.95 ml buffer 1 pH 8.8 (36.6 g tris base, 200 ml distilled H_2O), 0.75 ml distilled H_2O, 2.5µl tetramethylethylenediamine (TEMED), 50 µl ammonium persulphate 10%, 0.5 ml 10% sodium dodecyl sulphate (1gm SDS, 10ml distilled H_2O)] and the stacking gel (1.0 ml 30% acrylamide, 1.95 ml buffer 2 pH 6.8 (12g tris base, 200 ml distilled H_2O), 1.95 ml distilled H_2O, 4µl tetramethylethylenediamine (TEMED), 50 µl ammonium persulphate 10%, 0.5 ml 10% sodium dodecyl sulphate SDS). The resolving gel was poured between two assembled gel plates with separating thickness 1.0 mm. Distilled water was added over the resolving gel to prevent drying of its surface. Thereafter the gel was left for about 60 minutes at RT to ensure its polymerization. Subsequently the distilled water was discarded and stacking gel was added over the resolving gel. Immediately after the stacking gel was added the lane-dividing comb was added carefully to avoid introducing air bubbles into the gel, hence the gel was left for 30 minutes for the polymerization of the stacking gel.

For preparation of loaded samples, they were transferred from -20°C and thawed at RT then loaded after been boiled for 4 minutes with DTT loading buffer (20ml 1M tris-buffer pH 6.8, 2ml glycerol, 5g SDS, 1.54g dithioerythriol (DTT), 13mg bromophenol blue, volume was made up to 50 ml using distilled H_2O). The gel was run at 200 Volts for about 1.30 hours in running buffer pH 8.3 (1.515g tris base, 7.2g glycine, 0.5g SDS, 500ml distilled H_2O) half-filled electrophoresis chamber. Afterwards the gel was immersed in Coomassie Blue staining solution for about 5 minutes (400 ml methanol,

70ml acetic acid, 0.250g Coomassie Brilliant Blue R-75, volume made up to 1000 ml using distilled H_2O).

In order to visualise any proteins, the gel was destained in destain solution (400 ml methanol, 70 ml glacial acetic acid, volume was made to 1000 ml using distilled H_2O) for about 5 minutes.

3.4.5 Western blot

In order to perform Western blotting, the protein samples were first resolved in SDS PAGE gel electrophoresis as described before. After activation of polyvinylidene difluoride (PVDF) membrane (GE Healthcare, formerly Amersham Biosciences) by immersing it in methanol for about 2 minutes, the gel was sandwiched (1 sponge (cathode), 1 filter paper, gel, PVDF membrane, 1 filter paper, 1sponge (anode)) and placed in the electro blotting chamber filled with transfer buffer pH 8.3 (3g tris Base, 14.4g glycine, 0.4g SDS, 1000 ml distilled H_2O). The transfer was run at 30 Volts for about 3 hours. Afterwards the PVDF membrane was immersed in non-fat milk (5%) (2.5g non-fat milk, 50 ml TBS/Tween(8.28g NaCl, 2.42g tris, 0.47ml Tween 20, make volume to 1000ml using distilled water)) for 2 hours at room RT to block nonspecific binding sites.

The membrane was incubated overnight at 4^oC with primary antibody. The antibodies and dilutions are listed in **Table 3.**

Antibody	Dilution	Incubation time/T	Source	Company	Cat. No.
SPARC	1:500	Overnight at 4^{o}C	Mouse monoclonal	Santa cruz biotechnology	sc-73051
p53	1:500	Overnight at 4^{o}C	Rabbit polyclonal	Santa cruz biotechnology	sc-6243
GAPDH	1:5000	Overnight at 4^{o}C	Rabbit polyclonal	Abcam Cambridge, UK	ab9485
GAPDH	1:5000	Overnight at 4^{o}C	mouse monoclonal	Abcam Cambridge, UK	ab8245
p21	1:500	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab7960
catalase	1:2000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16731
MDM2	1:300	Overnight/ 4°C	Mouse monoclonal antibody	Santa cruz biotechnology	sc-965
MDMX	1:1000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16058
MDMX-phospho	1:1000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab70236
TGF-β1	1:500	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab66043
VEGF-A	1:1000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab46154

Table 3

List of primary antibodies used in Western blot.

All the previous antibodies were diluted in 5% w/v non-fat dry milk powder in TBS / Tween. This was followed by 3 washing steps within 30 minutes in TBS / Tween under continuous shaking. The membrane was transferred into a plastic dish containing one of secondary antibodies which was diluted in 5% w/v non-fat dry milk powder in TBS/Tween depending on the host of the primary antibody and incubated for 1 hour at RT, followed by wash in TBS/Tween **(Table 4)**. Positive immuno-reactivity was detected by the enhanced chemiluminescence's method (ECL). To do so, the membrane was immersed for 4 minutes in equal volumes of enhanced chemiluminescence's solution I (1 ml of 250 mM luminol in DMSO, 0.44 ml of 90 mM p-coumaric acid in DMSO, 10 ml of 1 M Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma, Pool, Dorset,UK) and solution Π (64 μl of 30% v/v H_2O_2, 10 ml of Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma, Pool, Dorset, UK). Positively stained protein bands send a luminescent signal which was visualised on a sheet of CL-XPosure Film (Thermo scientific, UK).

Stripping of membrane was taking place by washing the membrane for 30 minutes in TBS/Tween then it was exposed to restore western blot stripping buffer (Thermo scientific, UK) for 15 minutes, followed by washing three times in TBS/Tween, 10 minutes each. The rest of the experiment including blocking and incubation with primary and secondary antibodies and finally the developing process were exactly the same as described above.

Antibody	Dilution	Incubation time	Company	Cataloge number
Anti-Mouse IgG Peroxidase antibody produced in goat	1:1000	1h/RT	Sigma-Aldrich, St. Louis, USA	A2554-1ML
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	1:1000	1h/RT	Sigma-Aldrich, St. Louis, USA	A9169-2ML

Table 4

List of secondary antibodies used in Western blot.

Statistical analysis for Western blot

The bands were evaluated by utilizing Image J version 1.37 (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). Each band was highlighted and the intensity was measured. The mean of the calculated values was normalized and plotted in relation to the corresponding GAPDH using Microsoft Excel (Microsoft Corporation, USA). Statistical analysis was carried out by un-paired t-test.

3.5 Dot blot for SPARC

To study, whether H_2O_2 (30%, Fluka, Sigma, Pool, Dorset, UK) can affect SPARC binding affinity to the antibody, dot blotting technique was carried out, using recombinant human SPARC (R&D systems, UK). Per dot, 1ul of recombinant human SPARC was used at a concentration of 0.2µg/µl. SPARC was incubated with different concentration of H_2O_2 (0.5, 1, 5, 10, 50 and 100mM) for 1 hour at RT. Thereafter some dry paper towel was placed on the work surface, and then 4 filter papers, immersed in

0.05% tween 20 in TBS, were put on top of these paper towels, followed by addition of a PVDF membrane (GE Healthcare, formerly Amersham Biosciences, UK). The membrane, soaked in methanol for 2 minutes, washed in distilled H_2O and transferred to 0.05% tween 20 in TBS for 2-3 minutes. Protein samples were spotted in a row onto the PVDF membrane and allowed to dry for approximately 5 minutes at RT. Thereafter, the membrane was incubated in blocking solution containing 5% non-fat milk in TBS/Tween 20 [2.5g non-fat milk, 50 ml TBS/Tween (8.28g NaCl, 2.42g Tris, 0.47ml Tween 20, make volume to 1000ml using distilled water)] on a shaker for 2 hours at RT. Then the membrane was washed for 3 times with TBS/Tween, 10 minutes each, followed by incubation with the primary antibody (mouse monoclonal for SPARC, Abcam, Cambridge, UK) at the dilution of 1:500 overnight at 4°C. The rest of the experiment, including the washing procedures and incubation with the secondary antibody and finally the developing process were the same as described above (Western blot).

3.6 Fourier Transform (FT) -Raman spectroscopy

FT-Raman spectra were acquired using a BRUKER RFS 100/S spectrometer (Bruker, Karlsruhe, Germany) with a liquid-nitrogen-cooled Germanium detector. Near-infrared excitation was produced by a Nd^{3+}:YAG laser operating at 1064 nm. Each spectrum was accumulated over 17 min with 1000 scans and a resolution of 4 cm^{-1}. Detection of H_2O_2 is based on the O=O stretch at 875 cm^{-1}. This experiment was done in collaboration with Dr H. Rokos.

3. 6 Computer modelling of native, oxidised and nitrated SPARC

The crystal structure of SPARC was obtained from the protein data bank which is available online at: http://www.rcsb.org/pdb/home/home.do. Using the molecular modelling program "HyperChem" (Hypercube, Inc, Gainesville, FL, USA). The SPARC structure contains 4 methionine, 3 tryptophan and 7 tyrosine residues which are target to H_2O_2 / $ONOO^-$-mediated oxidation / nitration. DeepView analysis was applied to compare and analyse changes between the native and oxidised states of the protein (Dr Nicholas Gibbons is acknowledged for molecular modelling).

4. Results

4.1 Confirmation of low epidermal catalase expression, up-regulated p53, p21 in association with up-regulated $p76^{MDM2}$ in vitiligo as prerequisite for further investigation

4.1.1 Significantly decreased *in situ* catalase levels in the entire epidermis of patients with vitiligo in comparison to healthy controls

In 1991 Schallreuter et al. showed low catalase protein levels in both lesional and non - lesional skin of patients with vitiligo. The enzyme catalase plays a crucial role in protection against oxidative stress based on its function in degrading the reactive oxygen species H_2O_2 to O_2 and H_2O (Aronoff, 1965; Schallreuter et al., 1991). Due to accumulation of epidermal H_2O_2 in 10^{-3}M range, enzyme expression and functionality are severely affected, causing in turn degradation of its porphyrin ring as well as oxidation of target amino acids in the active site and in the teramerisation domain (Aronoff, 1965; Gibbons, et al., 2006; Maresca et al., 2006; Wood and Schallreuter, 2006). Later epidermal catalase protein expression has been introduced as a biomarker for oxidative stress in vitiligo (Salem et al., 2009). Therefore we decided to use this protein to prove the presence of oxidative stress *in situ* in patients with vitiligo (n=36: 6 patients, 6 pictures each) and healthy controls (n=36: 6 individuals, 6 pictures each) via immuno-fluorescence labelling. The results confirmed significantly lower catalase protein expression in the entire epidermal compartement in both lesional and non-lesional skin of the patients compared to normal controls. This down-regulated expression is also seen in the epidermal cell's nuclei of lesional and non-lesional skin compared to those of normal skin **(Figure 14)**. Image analysis of catalase staining intensity proves significantly decreased protein expression in both vitiligo lesional ($p<$

0.001, mean ±SE) and vitiligo non-lesional (p< 0.001, mean ±SE) throughout the entire epidermis compared to healthy controls **(Figure 15)**.

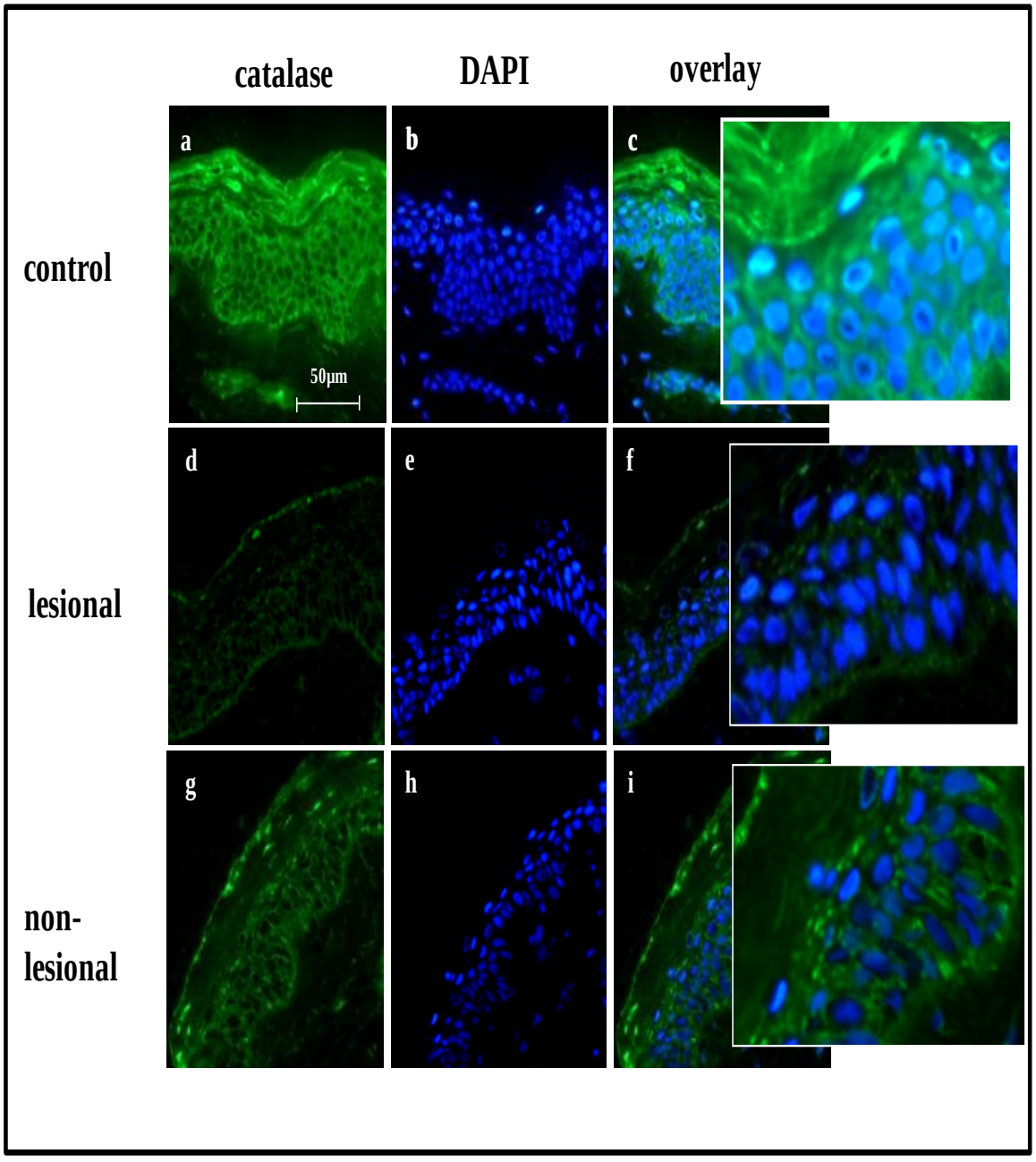

Figure 14: Low epidermal catalase expression in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows low expression of catalase in non-lesional (g) and almost absence in lesional (d) skin of the patients compared to controls (a) with skin phototype III (Fitzpatrick classification). Scale bar 50µm, Magnification x 400.

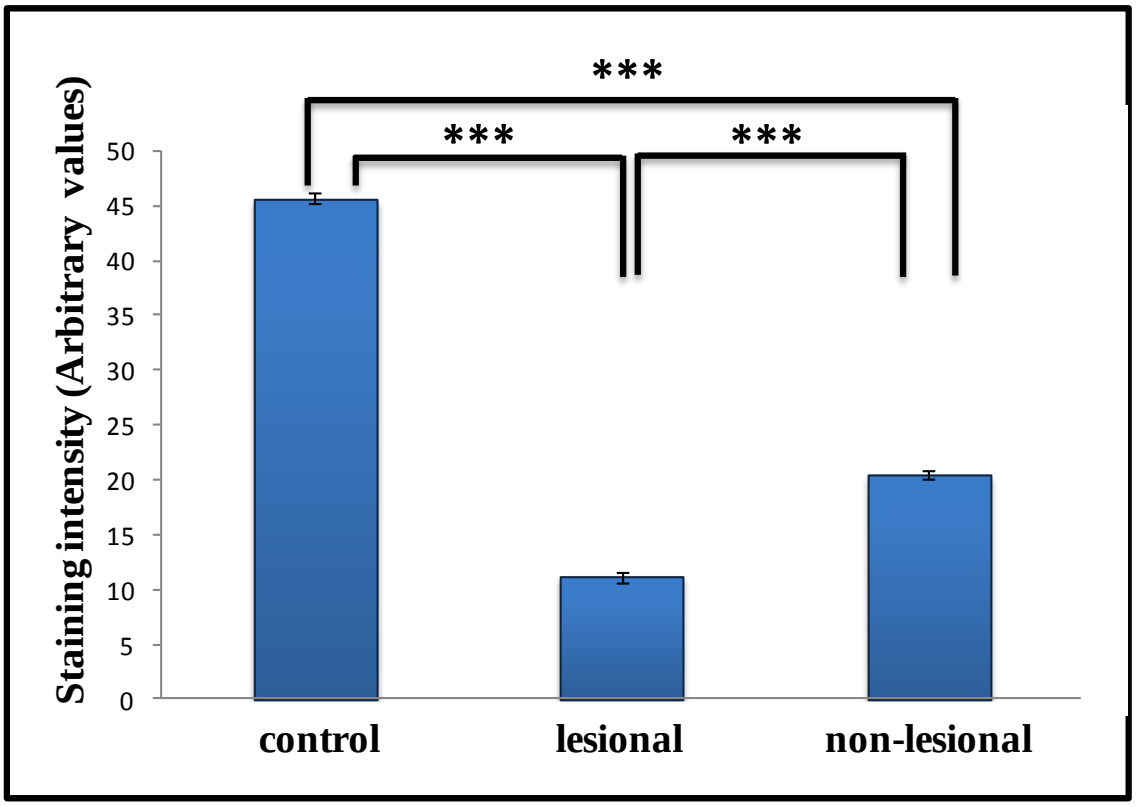

Figure 15: Significantly lower expression of epidermal catalase in vitiligo.

Image analysis of the average fluorescence intensity confirms significantly decreased catalase levels in patients lesional (n=36: 6 individuals, 6 pictures each) and non lesional (n=36: 6 individuals, 6 pictures each) skin compared to healthy controls (n=36: 6 individuals, 6 pictures each) (Plots are mean ± SE) (*** $p<0.001$).

4.1.2 Confirmation of low catalase levels in vitiligo by Western blot

In order to quantify catalase protein expression, we next employed Western blot. The results show decreased catalase expression in lesional (n=3) and non-lesional skin (n=3) of patients with vitiligo compared to skin of healthy controls **(Figure 16a)**. Image analysis of catalase protein bands in relation to loading control protein GAPDH confirms significantly decreased expression in the epidermis of patients **(Figure 16 b, c)**.

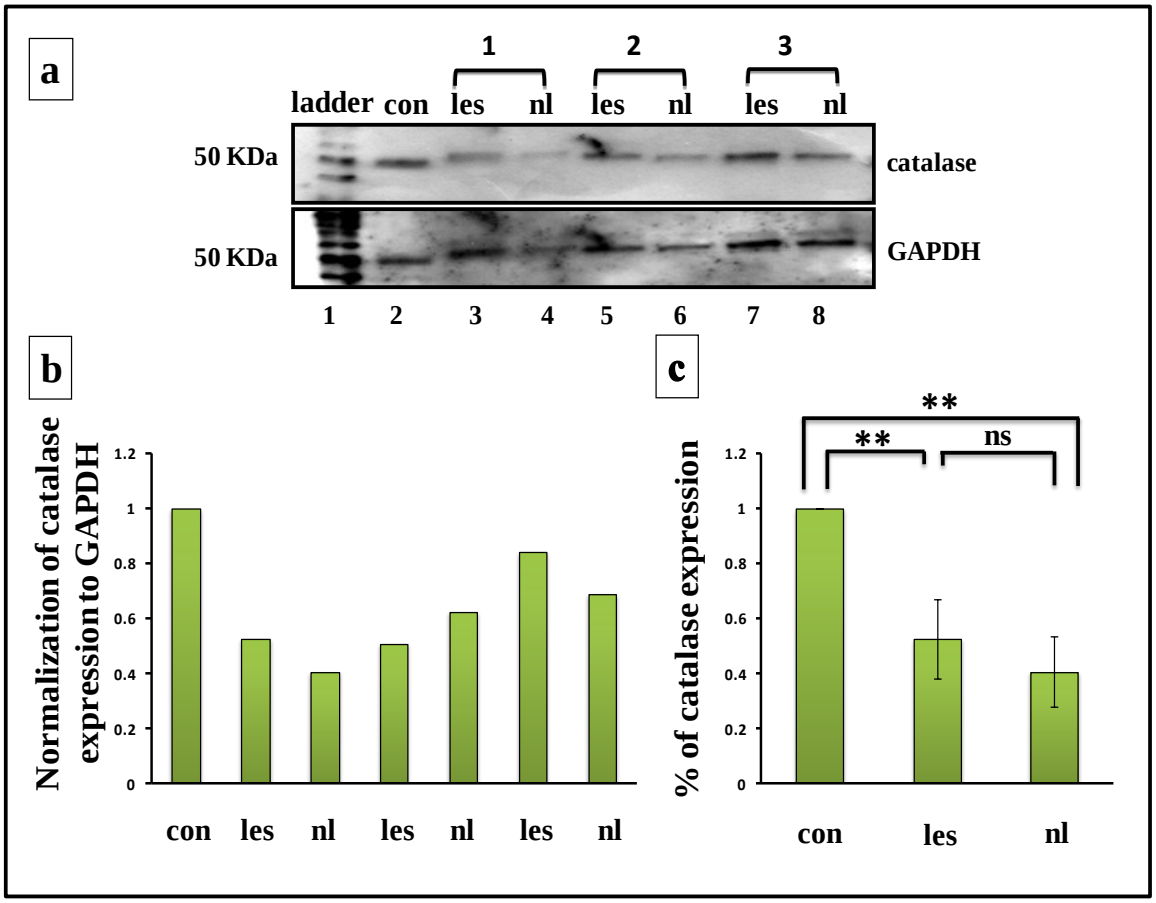

Figure 16: Significantly decreased epidermal catalase expression in skin of patients with vitiligo.

(a) Western blot. Catalase protein shows significantly decreased expression in both lesional and non-lesional epidermis of patients compared to healthy controls. Lane 1 protein ladder, lane 2 control, lanes 3-8 lesional and non-lesional skin tissue extracts from 3 patients. GAPDH was used as loading control.

(b) Normalization of catalase expression to the loading control. GAPDH was used to evaluate individual protein levels.

(c) Quantification of catalase bands. Image analysis was performed in relation to loading control protein (GAPDH). The result proves significantly decreased catalase expression in both lesional (n=3) and non-lesional (n=3) skin of patients compared to the control (n=1). (Plots are mean ± SE) (NS $p > 0.05$, ** $p<0.01$).

4.1.3 Catalase is present in epidermal melanocytes

After confirming decreased catalase expression throughout the entire epidermis of patients with vitiligo using both *in situ* imuno-fluorescence as well as Western blot, we looked at catalase expression and localization in normal and vitiliginous melanocytes under *in situ* conditions using double immuno-fluorescence with FITC-labelled catalase and TRITC-labelled NKI / beteb1. Melanocytes of non-lesional skin show almost absent catalase expression in the skin of patients with vitiligo, compared to control skin (**Figure 17).**

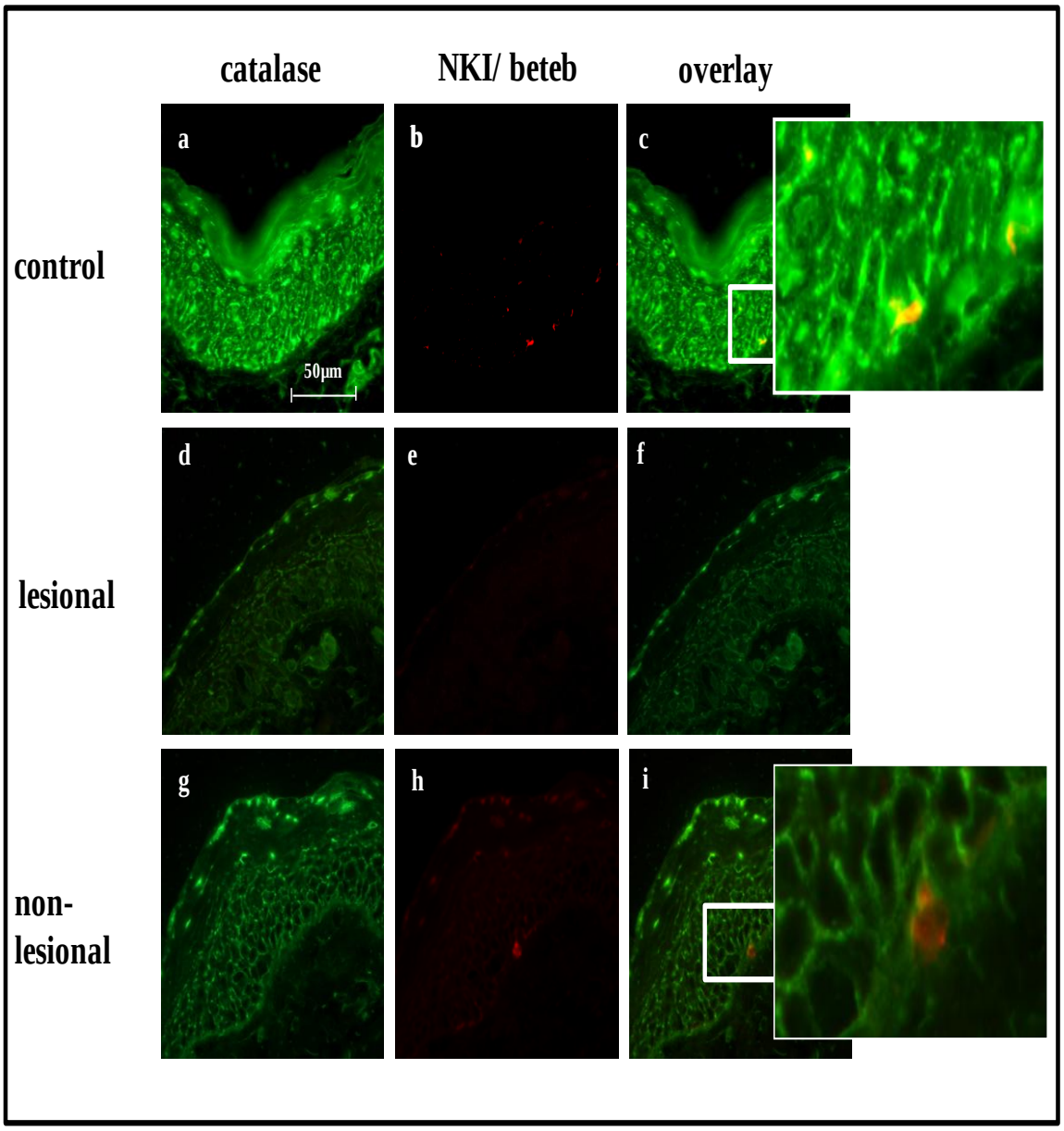

Figure 17: Decreased *in situ* catalase expression in epidermal melanocytes in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows decreased expression of catalase in lesional (d) and non-lesional (g) skin compared to control (a). Melanocytes are detected with TRITC-labelled NKI / beteb1. Overlay of NKI / beteb1 with catalase shows the presence of the enzyme in normal skin (yellow), but catalase expression is bearly detectable in melanocytes of non-lesional skin of patients. Scale bar 50µm. Magnification x 400.

Next, we looked at catalase expression under *in vitro* conditions. Unfortunately we had no melanocytes from patients with vitiligo. However, catalase expression is high in melanocytes of healthy controls. Expression is seen throughout the cell in the nucleus as well as in the cytoplasm. Strong co-localisation with NKI / beteb1 marks the presence in melanosomes (inserts) **(Figure 18).**

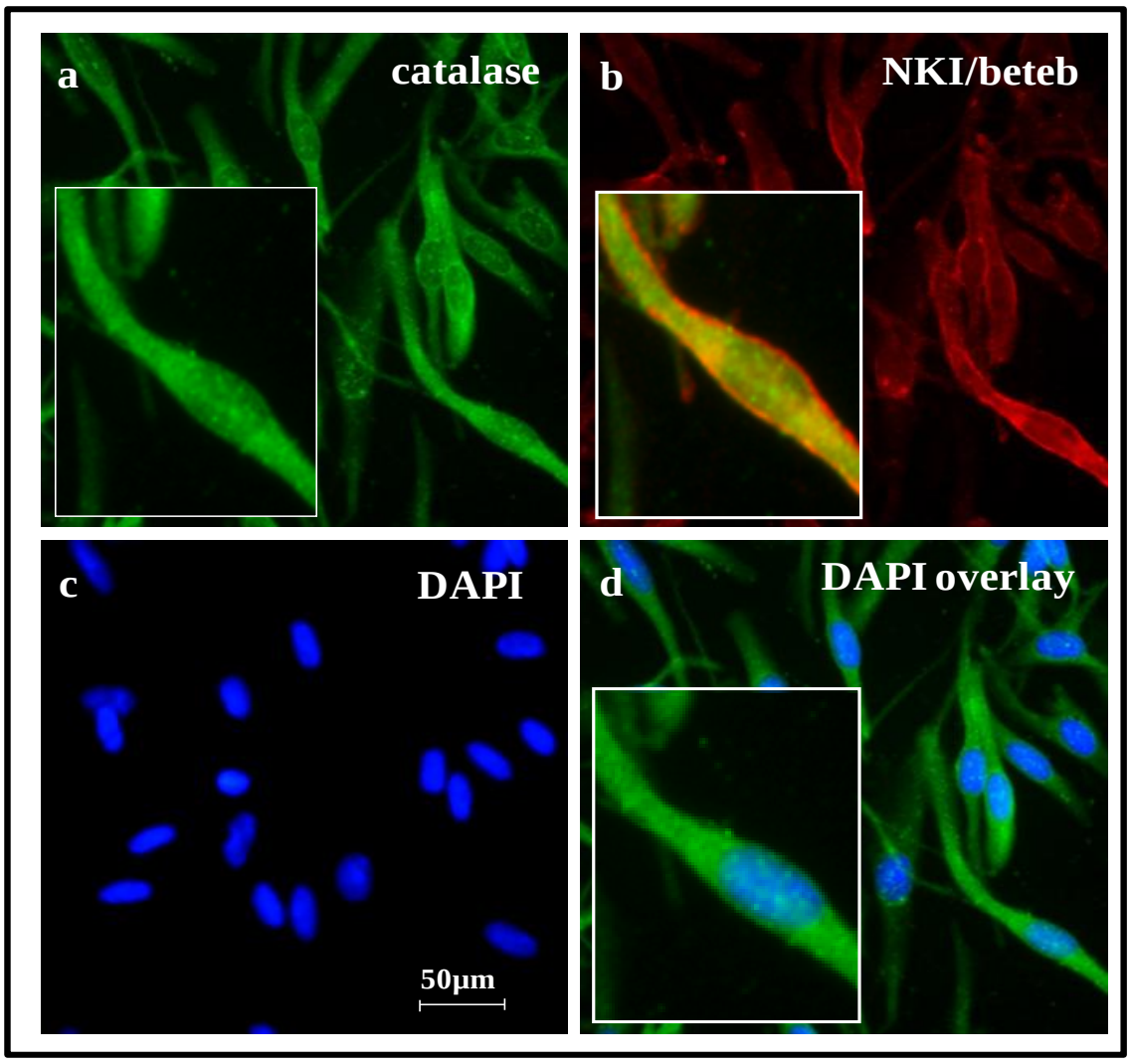

Figure 18: *In vitro* expression of catalase in normal human epidermal melanocytes.

Immuno-reactivity (FITC-labelling, green) reveals catalase expression in melanocytes. Patterns of expression indicate the presence of catalase in both, nucleus and cytoplasm (a, d and insert). Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled catalase shows strong yellow co-localisation of catalase in melanosomes (b and insert). Magnification x 400. Scale bar 50µm.

4.1.4 *In vivo* FT-Raman spectroscopy confirms the presence of 10^{-3}M H_2O_2 levels in the epidermis of patients with vitiligo

In order to confirm the presence of H_2O_2 in vitiligo, we utilised *in vivo* FT-Raman spectroscopy. The results show the expected peak at the O=O stretch at 875cm^{-1}. Based on these results we can conclude that H_2O_2 stress is present in the investigated patient samples **(Figure 19).** This result also confirms the influence of H_2O_2 on catalase protein expression, supporting in turn its role as a biomarker for evaluation of the epidermal H_2O_2 -mediated redox status in these patients (Gibbons et al., 2006, Schallreuter et al., 2013).

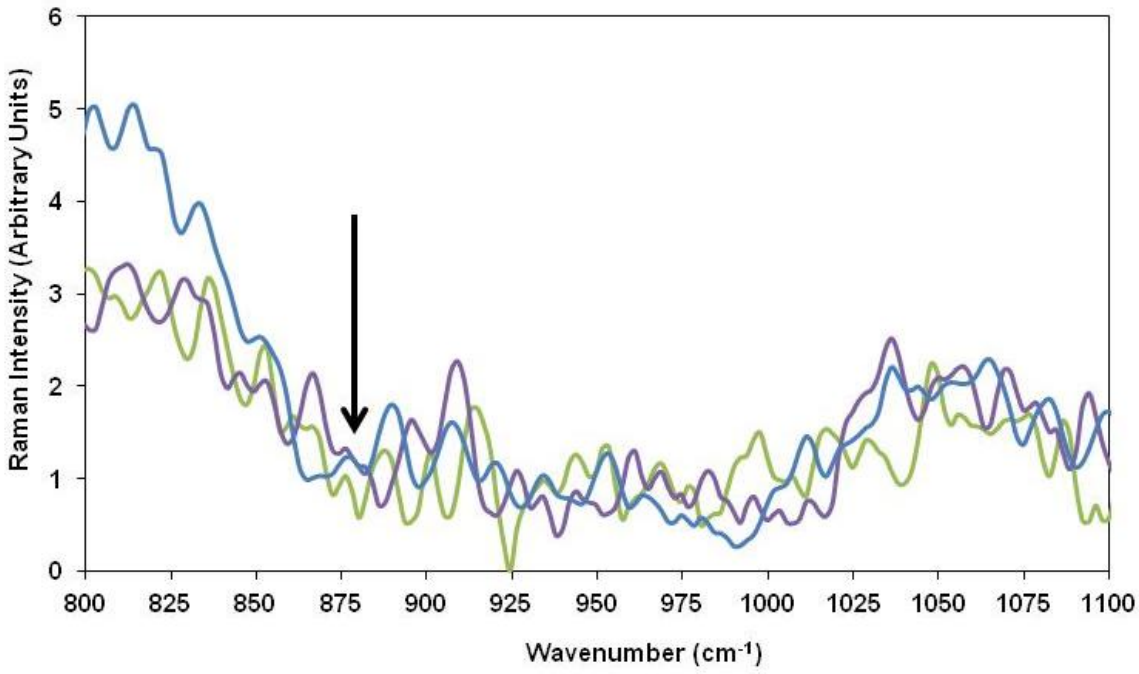

Figure 19: *In vivo* FT-Raman spectra from 3 patients with vitiligo.

The peaks at 875cm^{-1} are in agreement with the O=O stretch which is characteristic for H_2O_2. In this context it is noteworthy that detection of these peaks is only possible if the concentrations exceed 10^{-3}M.

In summary, we confirm the presence of H_2O_2- mediated stress in the 10^{-3}M range in the entire epidermal compartment by significantly lower epidermal catalase expression and protein levels in vitiligo samples and by *in vivo* FT-Raman spectroscopy as prerequisite for further studies.

4.1.5 The role of p53 in vitiligo

4.1.5.1 Significantly increased expression of epidermal p53 in the entire epidermis of patients with vitiligo

H_2O_2 up-regulates expression and function of wild type p53 (Vile, 1997; Salem et al., 2009). However, surprisingly, reduction of epidermal H_2O_2 with pseudocatalase PC-KUS does not affect the constant up-regulated expression of p53 protein in patients with vitiligo (Schallreuter et al., 2003; Salem et al., 2009). This result suggested that epidermal H_2O_2 levels were either still high enough to trigger p53 transcription or are regulated in a different way (Schallreuter et al., 2003). In this context it was suggested that up-regulated functioning p53 could serve as explanation of the non-elevated risk of skin photo-damage and non-melanoma skin cancer (basal-cell and squamous cell carcinomas) in these patients (Oettle, 1963; Calanchini- Postizzi and Frenk, 1987; Schallreuter et al., 2002; Salem et al 2009; Teulings et al, 2013).

Our *in situ* p53 results confirm those earlier results in vitiligo (Schallreuter et al., 2003; Salem et al 2009) by demonstrating significantly higher levels of p53 in lesioal (d) and non-lesional (g) skin throughout the entire epidermis compared to controls (a) **(Figure 20)**.

Image analysis of p53 proves significantly higher p53 protein expression in vitiligo lesional and non-lesional epidermis compared to healthy controls (plots are mean ± SE) (*** p<0.001, mean ±SE) (**Figure 21).**

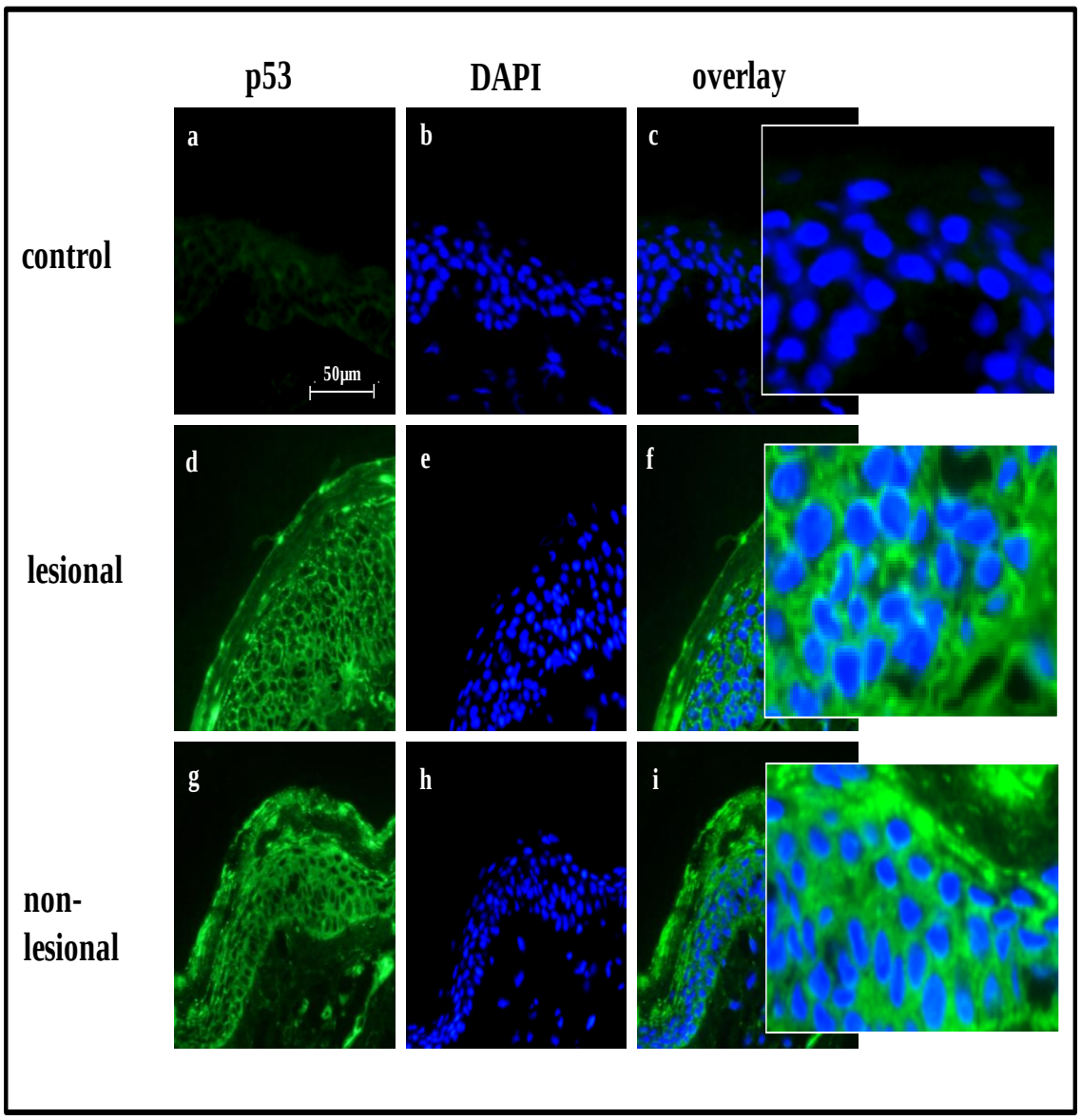

Figure 20: Higher epidermal expression of p53 in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows higher expression of p53 in lesional (g) and non-lesional (d) skin of patients throughout the entire epidermal compartment compared to controls with skin phototype III (a). Scale bar 50µm, Magnification x 400.

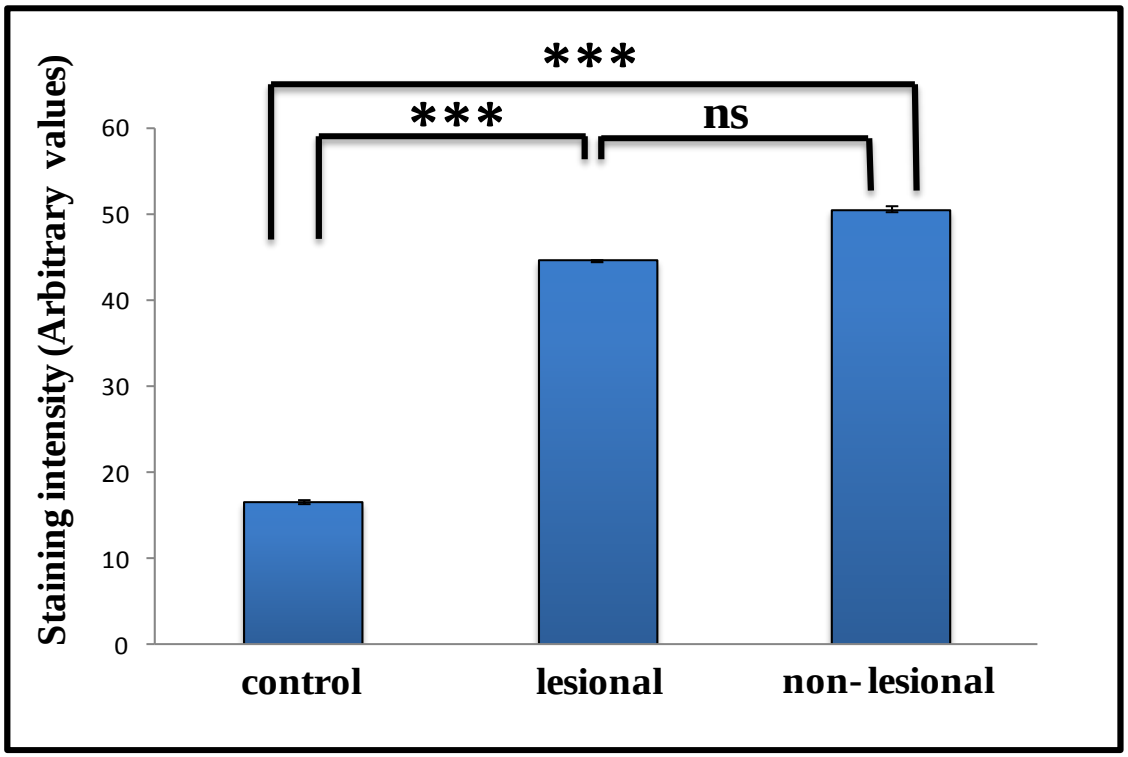

Figure 21: Significantly higher p53 expression in vitiligo.

Image analysis of average fluorescence intensity confirms significantly increased levels of p53 in lesional (n=18: 3 individuals, 6 pictures each) and non- lesional skin (n=18: 3 individuals, 6 pictures each) compared to controls (n=24: 4 individuals, 6 pictures each). There are no significant differences in expression between lesional and non-lesional skin of patients with vitiligo. (Plots are mean ± SE) (NS $p > 0.05$, *** $p<0.001$).

To further confirm up-regulated p53 expression, we employed Western blot. Once more the results document significantly up-regulated p53 expression in epidermal suction blister tissue from 3 patients in both lesional (n=3) and non-lesional skin (n=3) **(Figure 22a)**. Image analysis of p53 protein bands in relation to loading control protein (GAPDH) confirms significantly increased p53 expression in the entire epidermis of these patients **(Figure 22b, c)**.

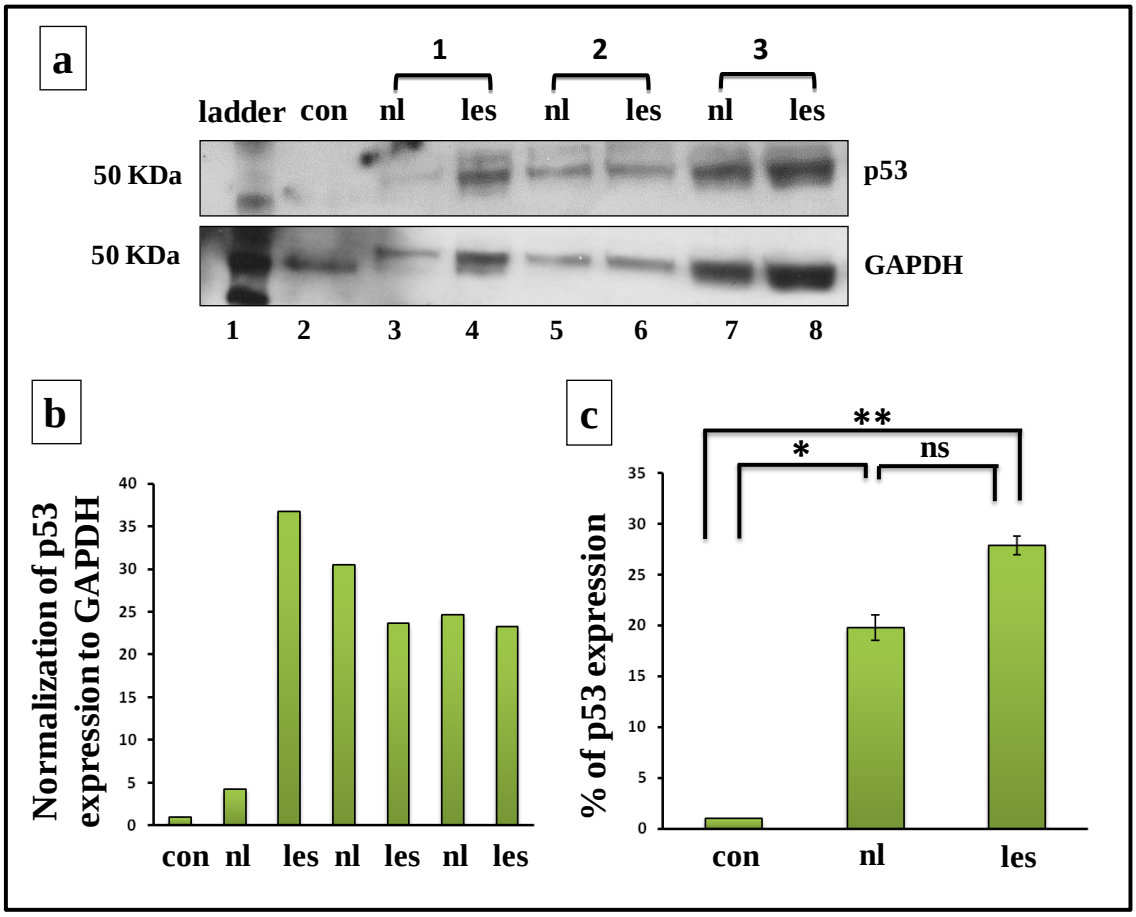

Figure 22: Significantly higher epidermal p53 levels in patients with vitiligo.

(a)Western blot of p53 protein (approximately 53 KDa) shows increased levels of its expression in both lesional and non-lesional skin of patients compared to control. Lane 1 protein ladder, lane 2 control, lanes 3-8 lesional and non-lesional skin tissue extracts from 3 patients. GAPDH was used as loading control.

(b) Normalization of p53 expression to the loading control. GAPDH was used to evaluate individual expression of the protein.

(c) Quantification of the p53 bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals significantly up-regulated expression in both lesional (n=3) and non-lesional (n=3) skin of patients compared to control (n=1). (Plots are mean ± SE) (NS $p>0.05$, * $p<0.05$, ** $p<0.01$).

4.1.5.2 p53 expression in vitiliginous melanocytes

As p53 is strongly expressed in the entire epidermis of patients with vitiligo, it was tempting to investigate the *in situ* expression in the target cell i.e. the melanocyte. Our *in situ* results show the presence of p53 in vitiliginous melanocytes of non-lesional skin. This protein is absent in healthy control skin **(Figure 23).**

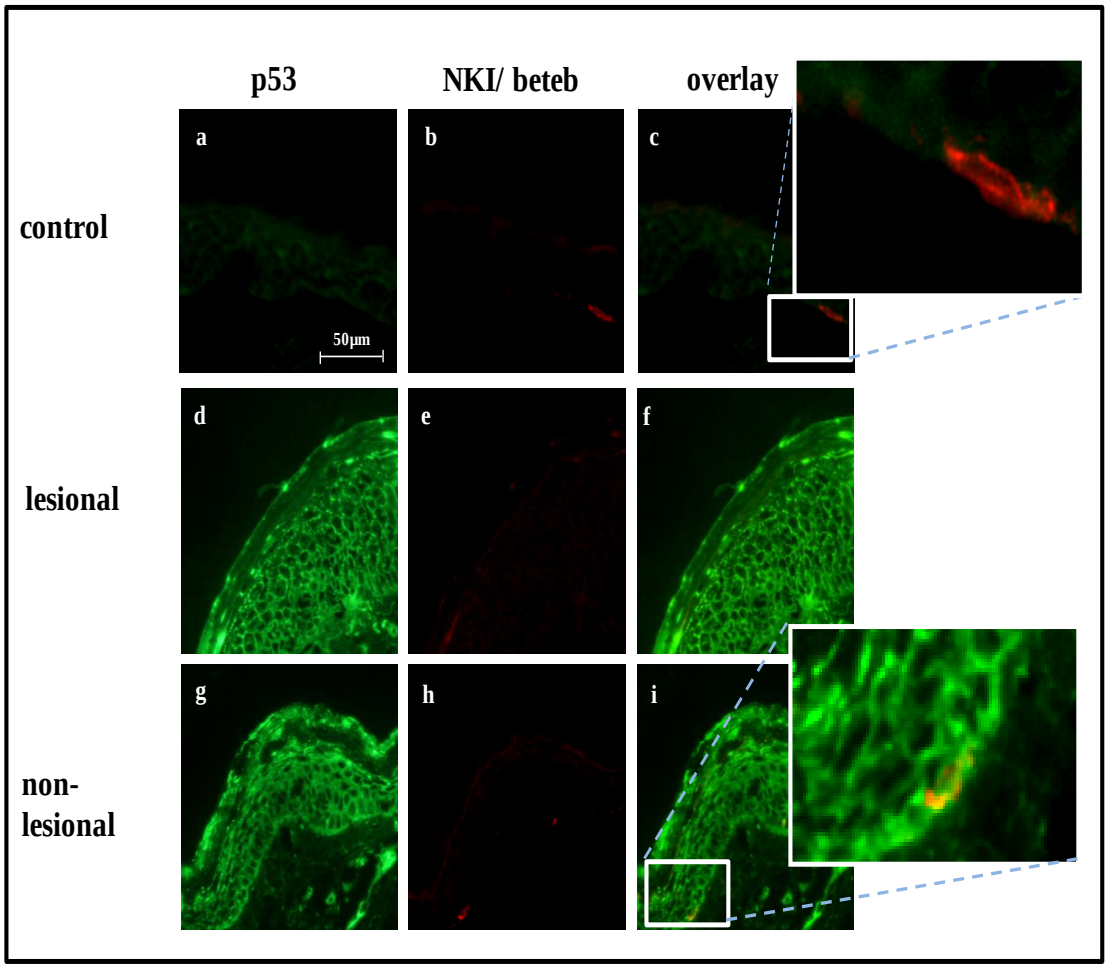

Figure 23: Increased p53 expression in epidermal melanocytes in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows higher expression of p53 in lesional and non-lesional (d, g) skin compared to healthy control (a). Intrabasal melanocytes are detected with TRITC-labelled NKI / beteb1. Overlay with p53 shows the presence of p53 protein in melanocytes (yellow) of non-lesional skin which is absent in control skin. Scale bar 50µm. Magnification x 400.

4.1.5.3 Significantly up-regulated *in situ* expression of p21 in both lesional and non-lesional skin of vitiligo compared to healthy control skin

The multifunctional protein p53 is mediating transcription of many proteins involved in cell cycle arrest as well as DNA-repair and apoptosis (Lane, 1992; Harris, 1993; Prives, 1994). One of these proteins, playing an important role in cell cycle arrest, is p21, interfering with cell cycle at the G1 phase (Kuerbitz et al., 1992; el-Deiry et al., 1993) and G2 phase (Lin and Lowe, 2001) providing in turn time for DNA repair (Hartwell, 1992; Hartwell and Kastan, 1994). In this context Salem et al were the first to show up-regulated p21 expression in vitiligo (Salem et al., 2009).

Here we confirm those earlier results, showing increased p21 expression throughout the entire epidermis of both lesional (d) and non-lesional (g) skin compared to controls (a) **(Figure 24**).

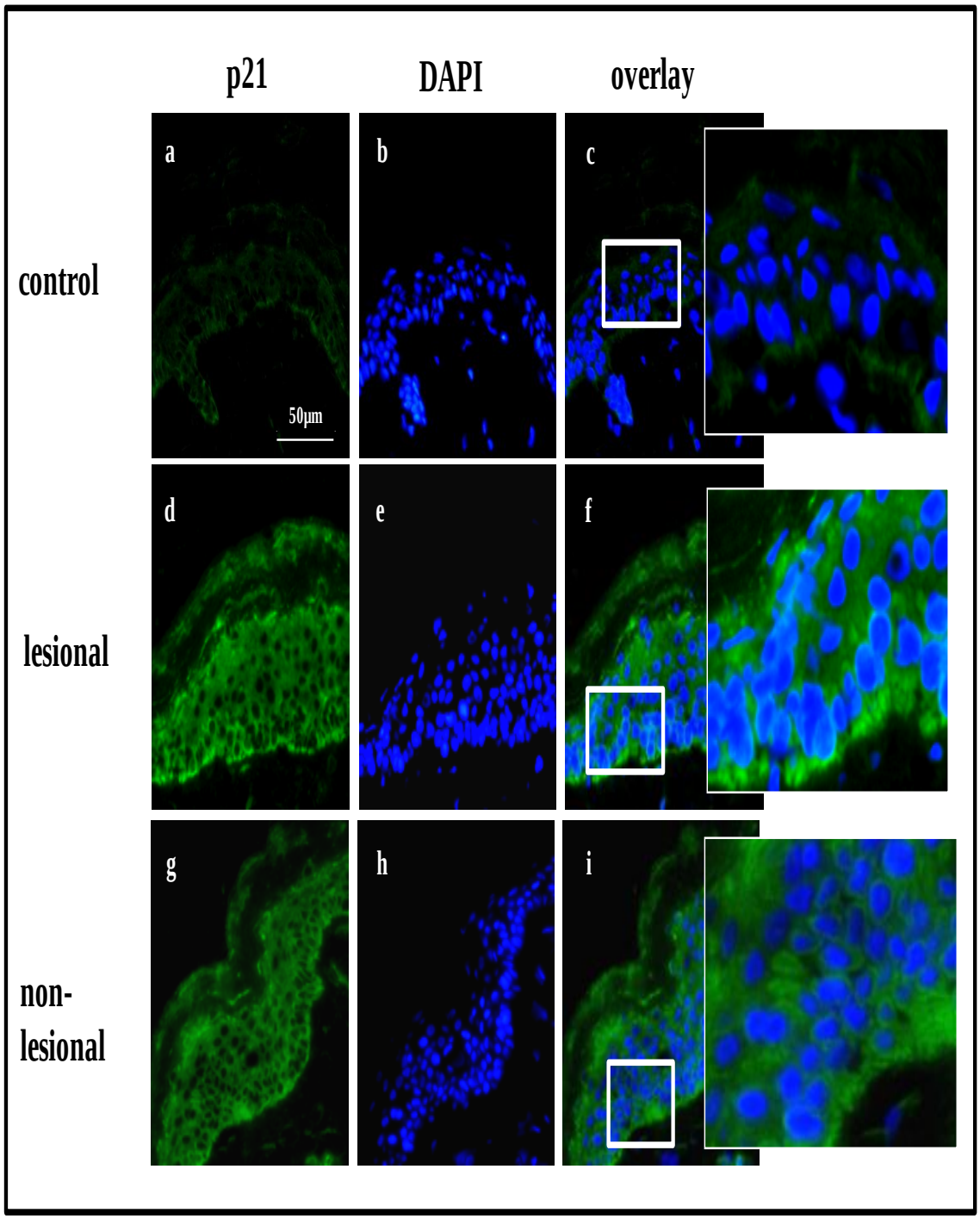

Figure 24: Up-regulated p21 expression throughout the entire epidermis in vitiligo.

Immuno-reactivity staining (FITC-labelling, green) shows increased p21 expression in lesional (g) and non-lesional (d) skin of patients compared to healthy control skin (skin phototype III) (a). Scale bar 50µm. Magnification x 400.

Image analysis of p21 confirms significantly higher protein expression in lesional and non- lesional epidermis compared to controls **(Figure 25)**.

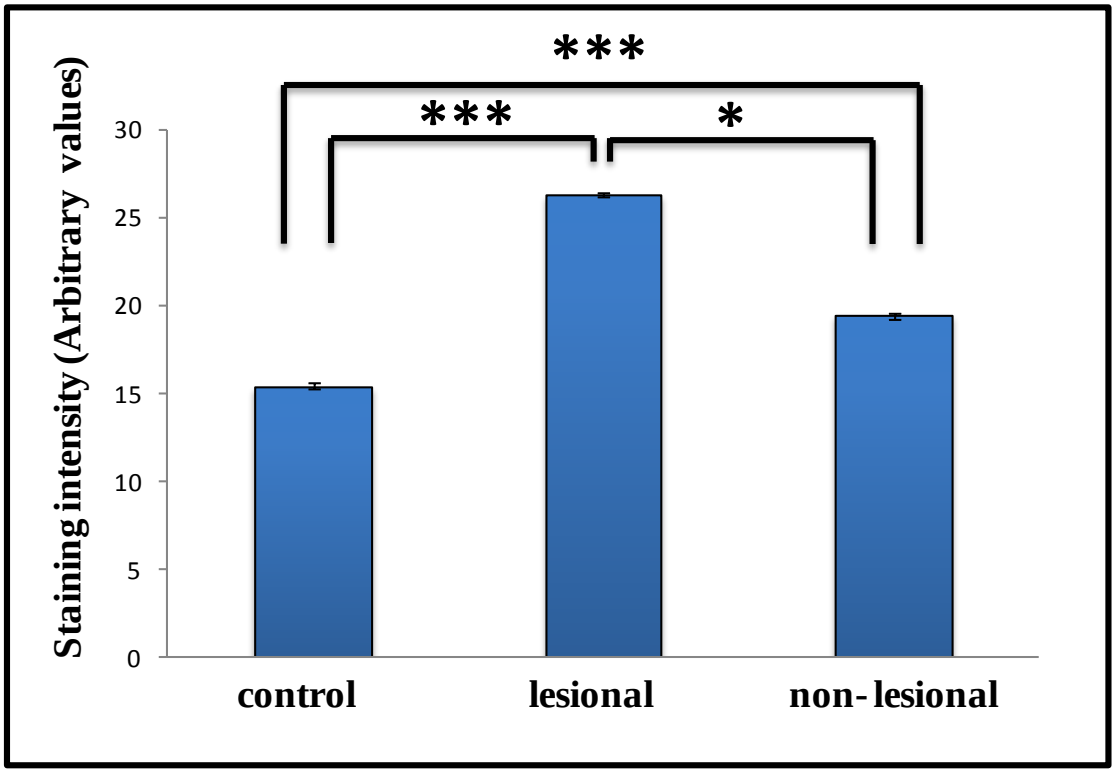

Figure 25: Significantly increased p21 expression in vitiligo.

Image analysis of **t**he average fluorescence intensity cofirms significantly increased protein levels of p21 in vitiligo lesional (n=24: 4 individuals, 6 pictures each) and non-lesional skin (n=24: 4 individuals, 6 pictures each) compared to healthy controls (n=18: 3 individuals, 6 pictures each). (Plots are mean ± SE) (*** $p<0.001$, * $p>0.05$).

In order to quantify protein expression, we used Western blot. The results confirm up-regulated p21 expression in lesional (n=3) and non-lesional skin (n=3) compared to skin of controls **(Figure 26a)**. Image analysis of p21 protein bands in relation to loading control protein GAPDH reveals significantly higher p21 expression in patient's skin **(Figure 26 b, c)**.

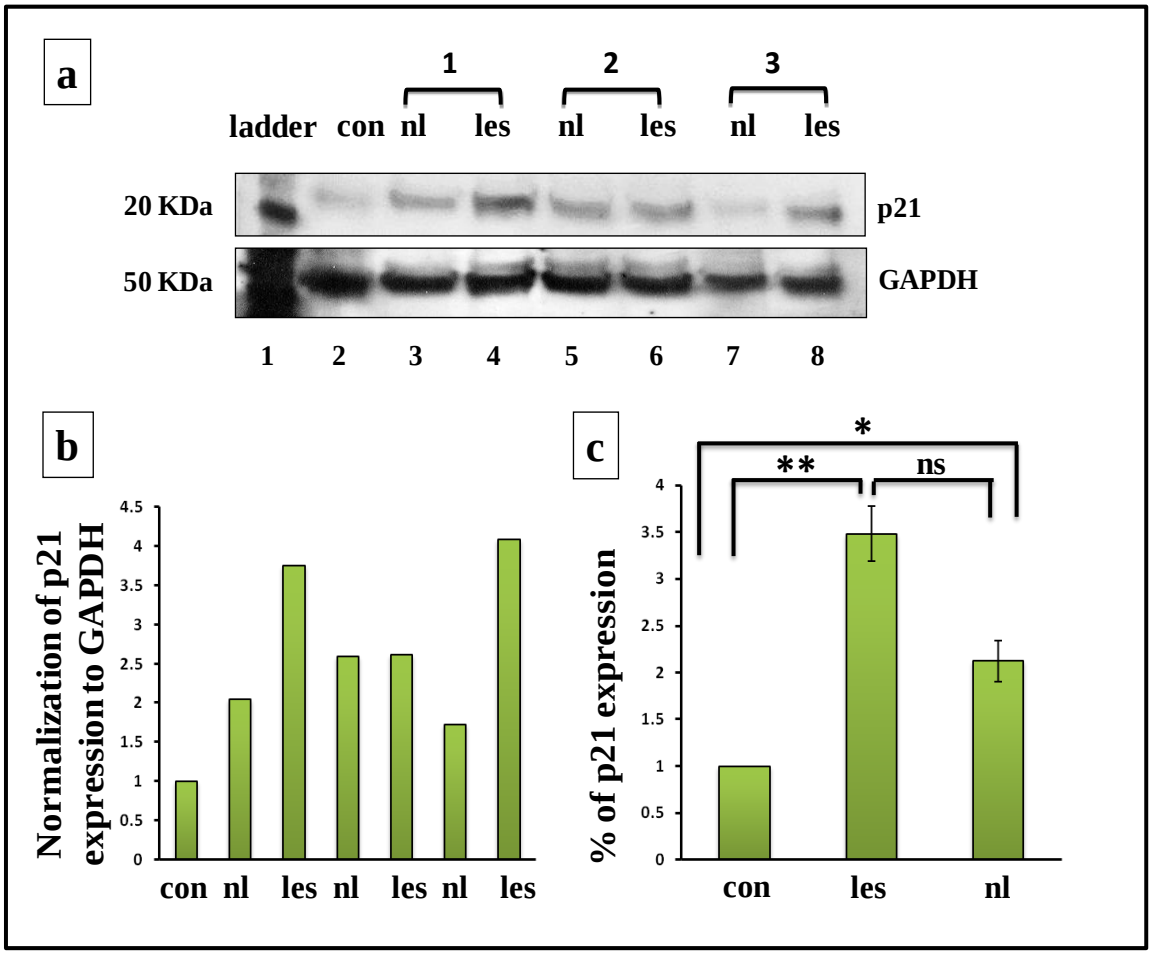

Figure 26: Significantly up-regulated p21 expression in skin of patients with vitiligo.

(a) Western blot. p21 protein shows significantly increased p21 levels in both lesional and non-lesional skin of patients compared to healthy controls. Lane 1 protein ladder, lane 2 control, lanes 3-8 non- lesional and lesional skin extracts from 3 patients. GAPDH was used as loading control.

(b) Normalization of p21 expression to the loading control. GAPDH was used to evaluate the individual protein expression.

(c) Quantification of the p21 bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals up-regulated expression in lesional (n=3) and non-lesional (n=3) skin of patients with vitiligo compared to the control (n=1). (Plots are mean ± SE) (NS $p>0.05$, * $p<0.05$, ** $p<0.01$).

4.1.5.4 Increased *in situ* p21 expression in melanocytes in vitiligo

In order to show the presence of p21 protein expression in epidermal melanocytes of patients, we utilised double immuno-fluorescence labelling with FITC - labelled p21 and TRITC - labelled NKI / beteb1 in full skin biopsies from healthy controls as well as patients. The *in situ* results show detectable p21 *in situ* expression (yellow) in melanocytes (**Figure 27)**.

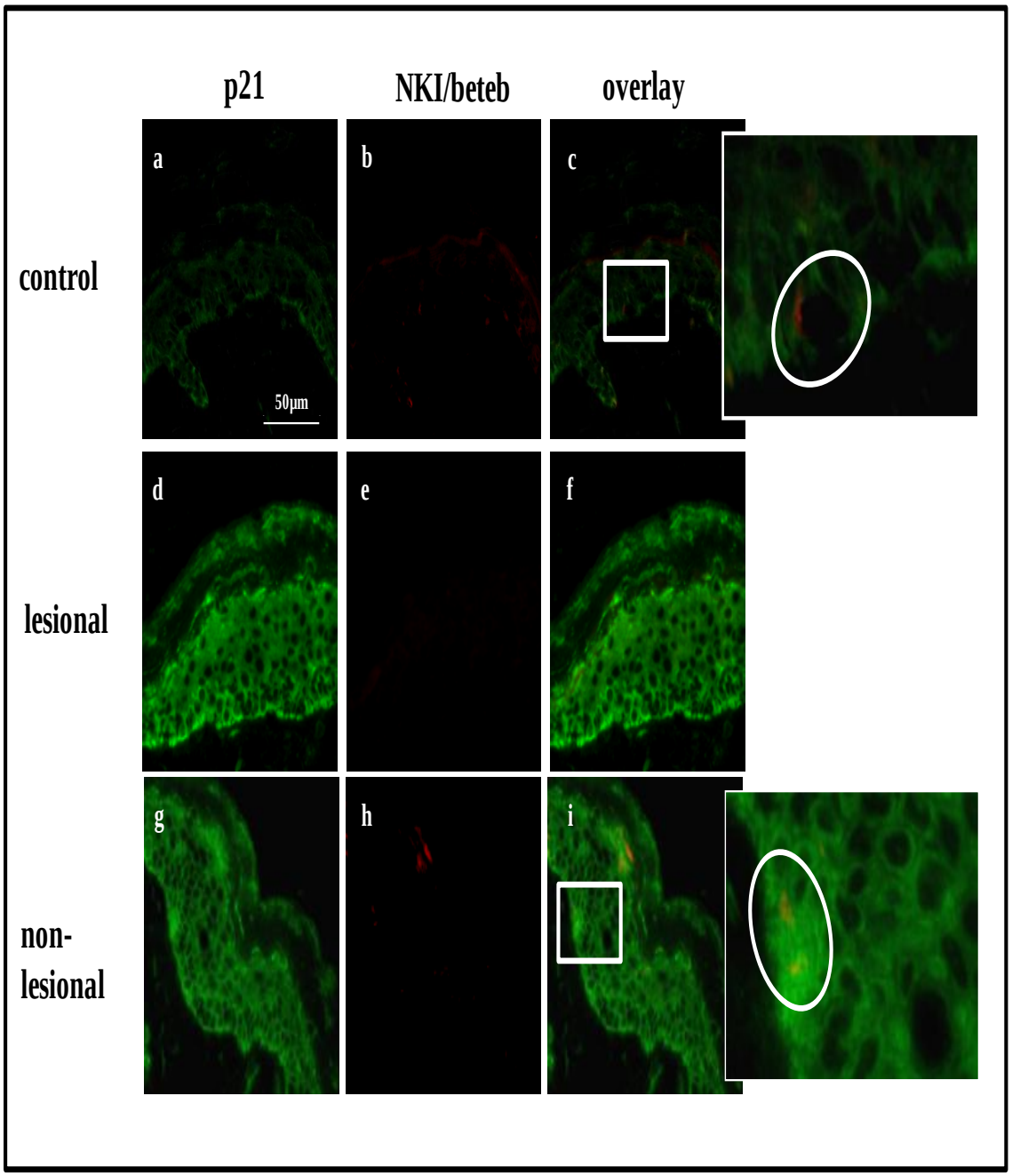

Figure 27: Increased p21 expression in epidermal melanocytes in vitiligo.

Immuno-reactivity (FITC- labelling, green) shows higher expression of p21 in lesional and non lesional (d, g) skin compared to healthy controls (a). Melanocytes are detected with TRITC- labelled NKI / beteb1. The overlay with p21 shows the presence of p21 protein in melanocytes (yellow) of non-lesional skin which is absent in healthy controls. Scale bar 50µm. Magnification x 400.

4.1.6 The effect of the MDM2 family on epidermal p53 levels, activity and stability in skin of patients with vitiligo

4.1.6.1 A view at p53 regulatory factors, including, $p76^{MDM2}$ and MDM4

$p90^{MDM2}$ and MDM4 are homologous proteins in control of p53 levels. Briefly, $p90^{MDM2}$ controls p53 activity via two distinct mechanisms. On the one hand, binding to the N-terminal domain of p53, prevents in turn binding of gene expression inducing factors causing a stop in the p53-dependent transcriptional machinery. On the other hand, it leads to ubiquitination of p53 by targeting the protein for proteasomal degradation (Haupt et al., 1997; Honda and Yasuda, 2000; Kubbutat et al., 1997; Oliner et al., 1993). MDM4 stabilizes $p90^{MDM2}$ by interfering with $p90^{MDM2}$ auto-ubiquitination. However, it has also been reported that MDM4 is ubiquitinated and degraded by $p90^{MDM2}$ (Pan et al., 2003). In this context it is of note that the smaller MDM2 protein $p76^{MDM2}$ acts positively towards p53 via antagonising the ability of $p90^{MDM2}$ to target p53 protein degradation (Perry et al., 2000; Giglio et al., 2010). As shown above epidermal p53 expression is high in vitiligo. These high levels were attributed to continuous oxidative stress, causing phosphorylation of p53 which in turn destabilizes the interaction between p53 and $p90^{MDM2}$, resulting then in protection of p53 from degradation (Salem et al., 2009). In this context it is noteworthy, that the same authors showed for the first time significantly elevated $p76^{MDM2}$ levels in the skin of patients with vitiligo in association with enhanced $p53^{w//w}$ functional activity. Based on these results a crucial role in control of DNA damage / repair, prevention of photo-damage and non-melanoma skin cancer in vitiligo was proposed (Salem et al., 2009). In response to UV- radiation MDM4 was found to be phosphorylated by CHK1, increasing in turn the binding ability to 14-3-3γ, leading then to its retention in the cytoplasm. The overall result is activation of p53 transcriptional function and G1 cell cycle arrest. Importantly, this p53 activation

is accompanied with both, an increased stabilization and decreased ubiquitination, suggesting that cytoplasmic phosphorylated MDM4 (MDM4S367P) inhibits MDM2 ubiquitin ligase function (Jin et al, 2006).

To get a better understanding for possible involved mechanisms behind p53 accumulation in the epidermis of patients with vitiligo, it was tempting to study MDM family members as p53-regulatory factors including $p76^{MDM2}$, MDM4 and MDM4phospho.

4.1.6.1.1 Up-regulated epidermal $p76^{MDM2}$ expression in vitiligo

Our *in situ* MDM2 immuno-fluorescence staining in vitiligo shows significantly higher levels of $p76^{MDM2}$ splice variant throughout the entire epidermis of lesional and non-lesional skin compared to controls. This up-regulated expression seems to be present also in the nuclei of epidermal cells **(Figure 28)**. Image analysis reveals significantly higher $p76^{MDM2}$ protein expression in lesional and non-lesional epidermis compared to control **(Figure 29)**.

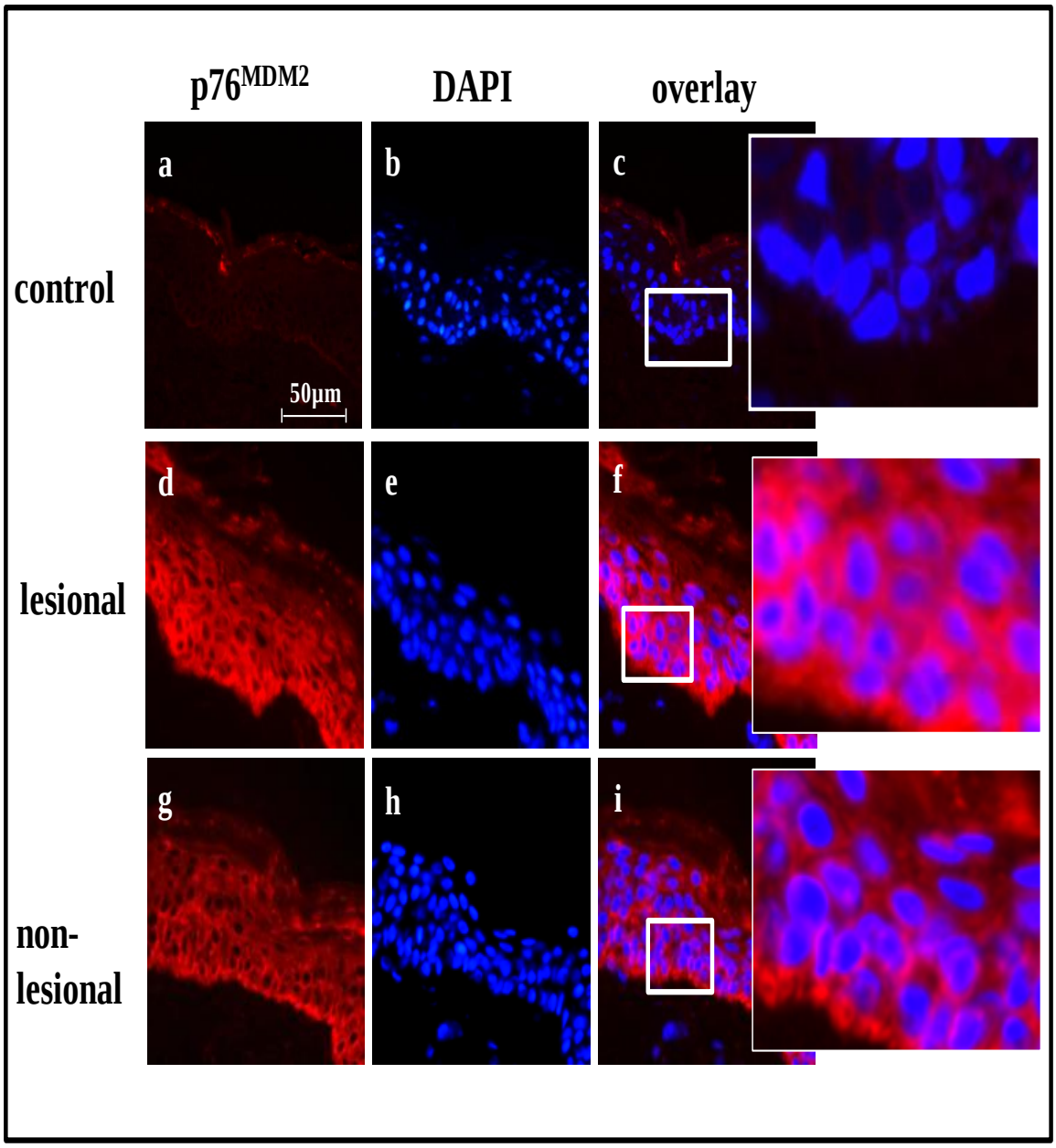

Figure 28: Up-regulated epidermal expression of p76^{MDM2} in vitiligo.

Immuno-reactivity staining (TRITC-labelling, red) shows increased expression of p76^{MDM2} throughout the epidermis of both lesional (d) and non-lesional (g) skin compared to controls with skin phototype III (a). Scale bar 50µm. Magnification x 400.

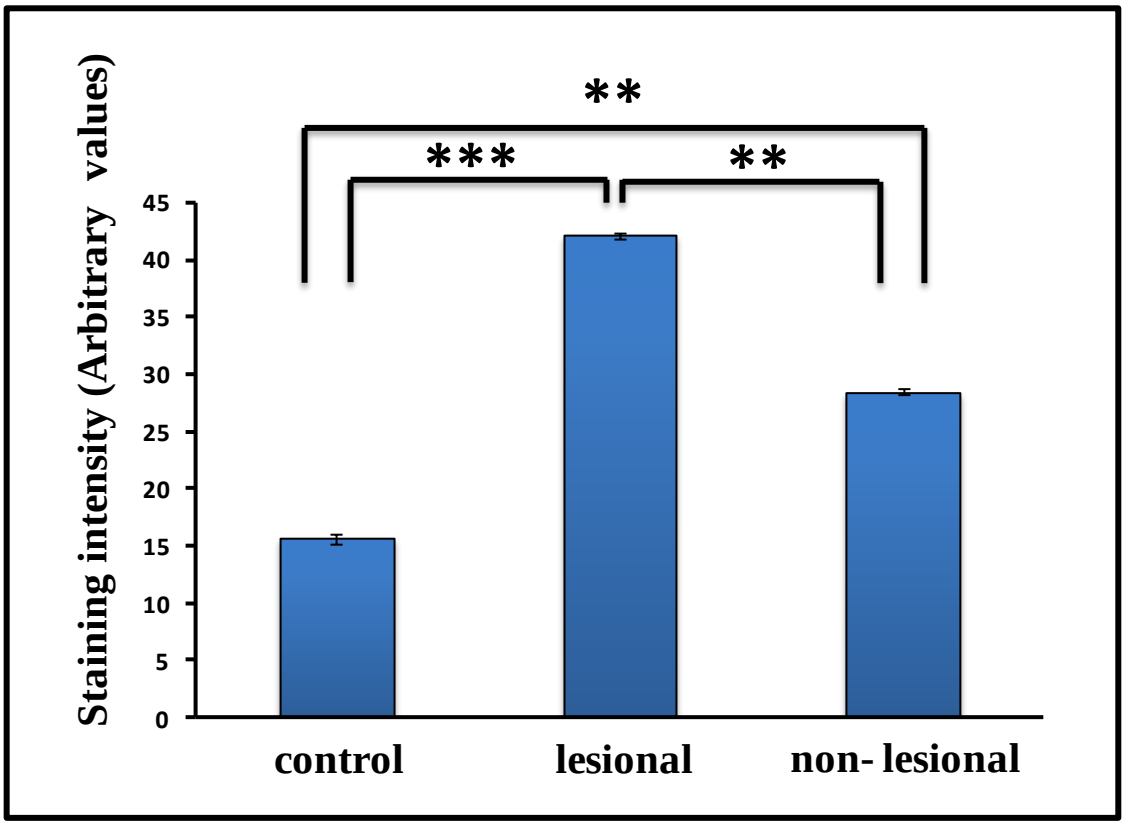

Figure 29: Significantly increased p76^{MDM2} expression in skin of patients with vitiligo.

Image analysis of the average fluorescence intensity shows significantly increased levels of p76^{MDM2} in the epidermis of lesional (n=30: 5 individuals, 6 pictures each) and non-lesional (n=30: 5 individuals, 6 pictures each) skin of patients with vitiligo compared to controls (n=18: 3 individuals, 6 pictures each). (Plots are mean ± SE) (** $p<0.01$, *** $p<0.001$).

4.1.6.1.2 Confirmation of elevated epidermal p76 ^{MDM2} in vitiligo by Western blot

Western blot was used to quantify $p76^{MDM2}$. Our results reveal significantly up-regulated $p76^{MDM2}$ in lesional and non-lesional epidermal suction blister tissue extracts compared to controls **(Figure 30 a, b)**.

In summary, both immuno-fluorescence and Western blot results were in agreement with previous data of the Schallreuter group (Salem et al., 2009), who reported significantly increased expression of $p76^{MDM2}$ in both lesional and non-lesional skin of patients with vitiligo. As they showed no differences in $p90^{MDM2}$ expression between vitiligo and controls, we did not include this protein in our study.

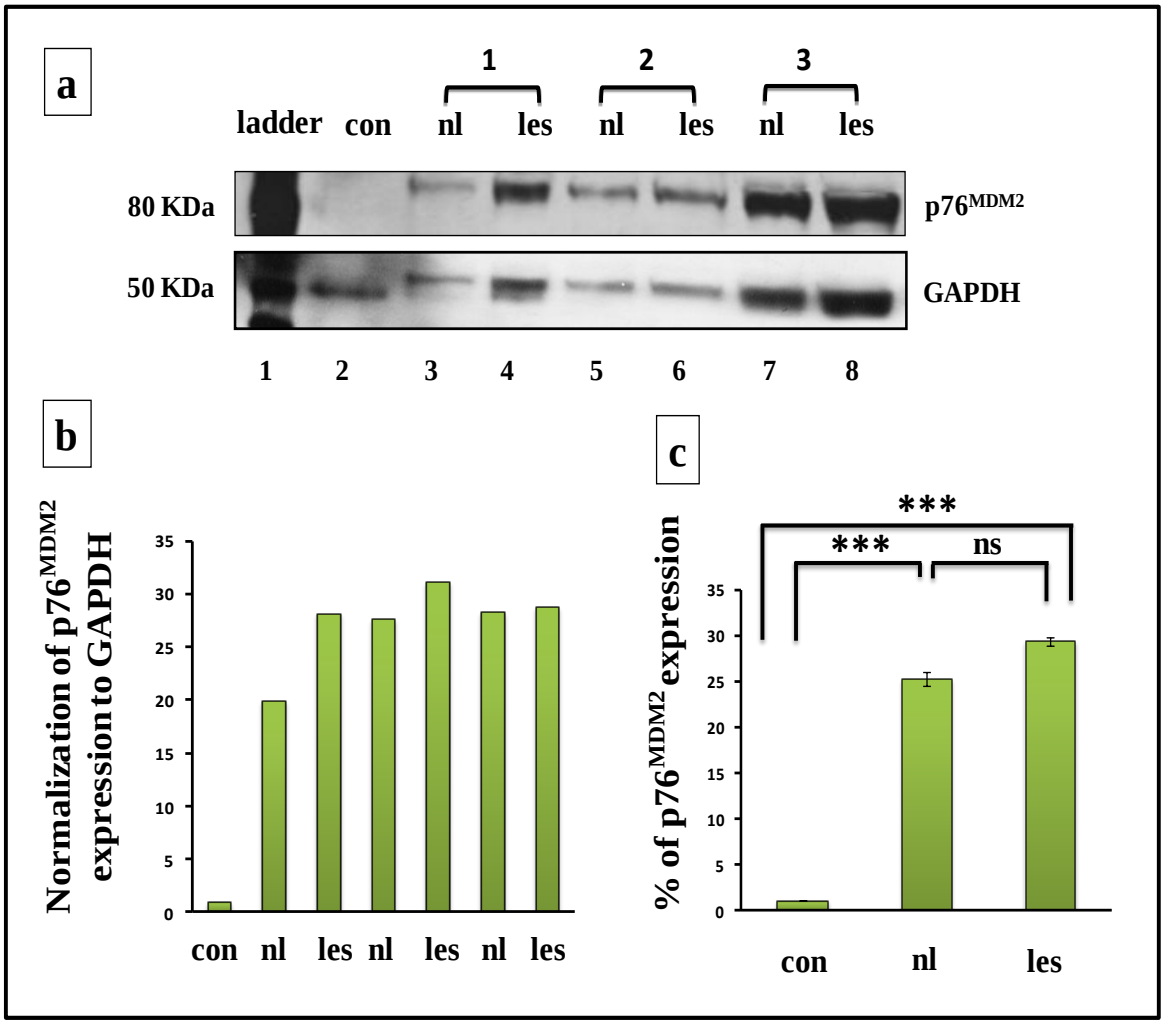

Figure 30: Significantly up-regulated p76^{MDM2} expression in skin of patients.

(a) Western blot for p76^{MDM2} shows up-regulated expression in lesional and non-lesional skin of patients compared to controls. Lane 1 protein ladder, lane 2 control, lanes from 3-8 lesional and non-lesional epidermal suction blister tissue extracts from 3 patients. GAPDH was used as loading control.

(b) Normalization of expression to loading control. GAPDH was used for evaluation of individual protein expression.

(**c) Quantification of p76^{MDM2} expression.** Image analysis of bands was used to quantify the expression of p76^{MDM2} protein in skin of patiets with vitligo in relation to the expression in control skin. The result confirms up-regulated p76^{MDM2} expression in lesional (n=3) and non-lesional (n=3) skin compared to the control (n=1). (NS $p > 0.05$, *** $p<0.001$).

4.1.6.1.3 *In situ* MDM4 and MDM4phospho expression in vitiligo

After characterisation and confirmation of previous results, we turned our interest to a possible role of MDM4 in controlling p53 in vitiligo. For this purpose we studied expression of both MDM4 and its phosphorylated protein (MDM4phospho) in patients and compared those results with healthy controls.

4.1.6.1.3.1 Significantly increased MDM4 expression in the entire epidermal compartment of patients with vitiligo

Our *in situ* results from MDM4 immuno-fluorescence labelling show higher levels of MDM4 throughout the entire epidermis in lesional and non-lesional skin of patients. Overlay of FITC-labelled MDM4 with DAPI suggests MDM4 expression in nuclei of epidermal cells **(Figure 31)**. Image analysis of MDM4 indicates significantly up-regulated MDM4 protein expression in lesional and non- lesional epidermis compared to controls **(Figure 32)**.

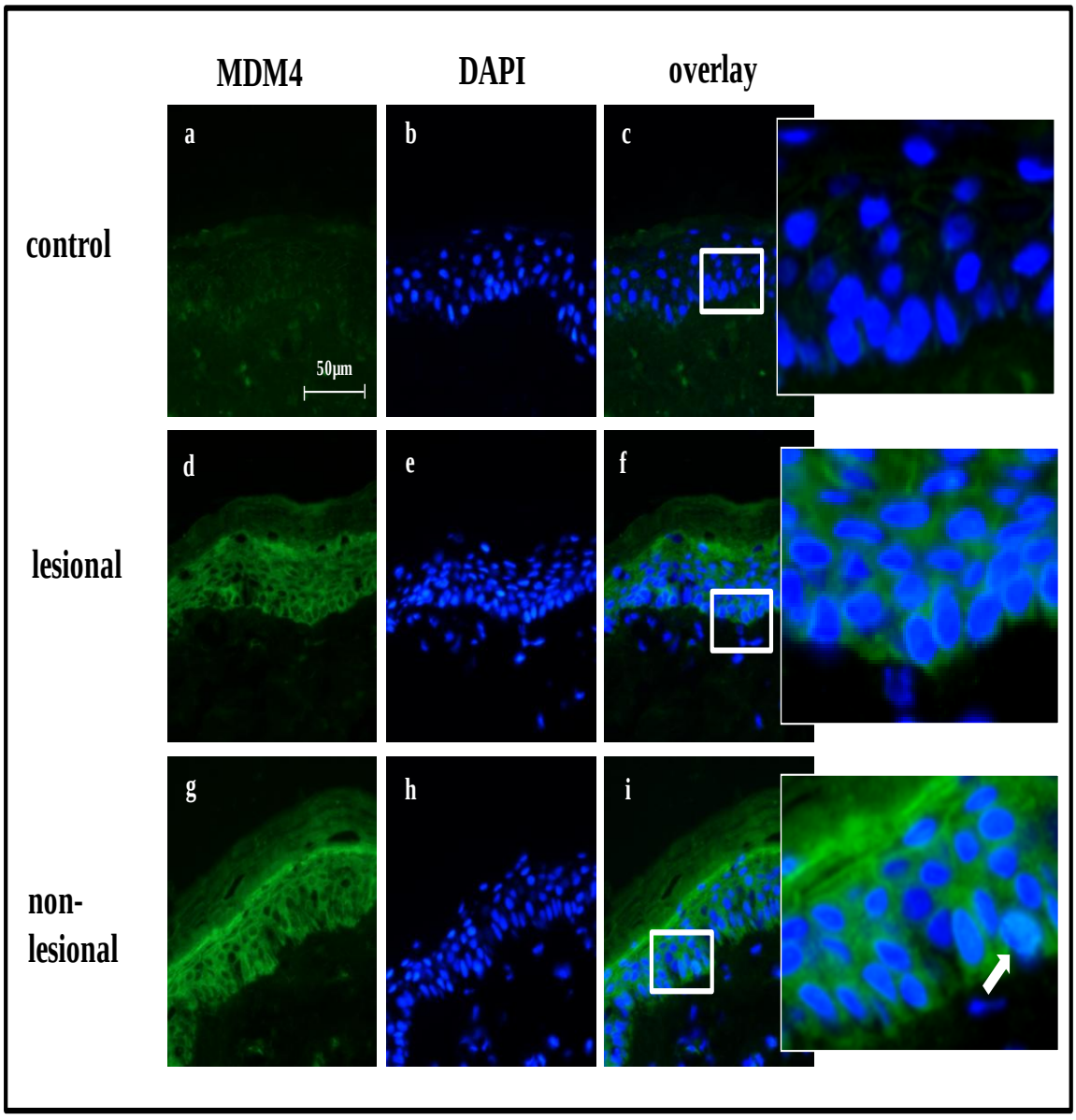

Figure 31: Significantly higher expression of MDM4 in vitiligo.

Immuno-reactivity staining (FITC-labelling, green) shows high expression of MDM4 in lesional (g) and non- lesional (d) skin of patients compared to the control with skin phototype III (a). MDM4 seems to be present in nuclei as shown by overlay with DAPI. Scale bar 50µm. Magnification x 400.

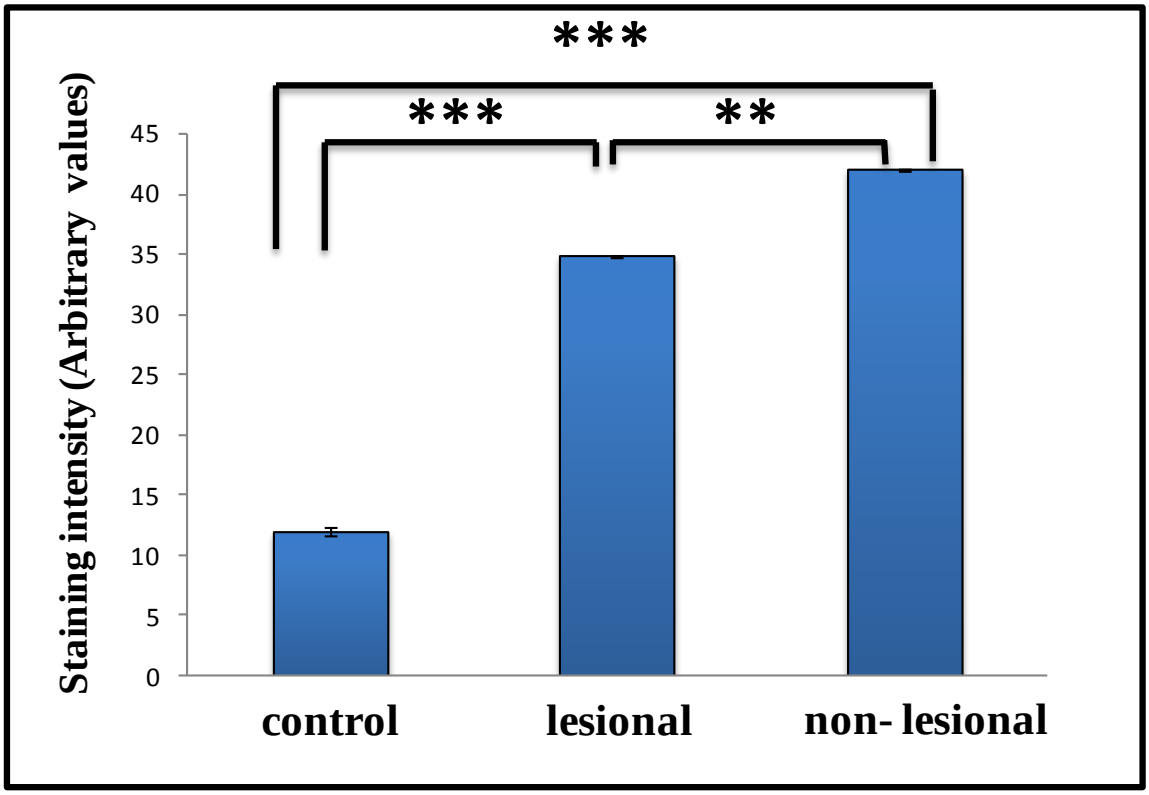

Figure 32: Significantly increased expression of MDM4 in vitiligo.

Image analysis of the average fluorescence intensity shows significantly up-regulated levels of MDM4 in vitiligo patients lesional (n=45: 5 individuals, 9 pictures each) and non-lesional (n=40: 5 individuals, 8 pictures each) skin when compared to healthy controls (n=27: 3 individuals, 9 pictures each). (Plots are mean ± SE) (*** $p<0.001$, ** $p<0.01$).

4.1.6.1.3.2 Confirmation of up-regulated MDM4 expression in lesional skin of patients with vitiligo by Western blot

In order to quantify protein expression, Western blot for MDM4 was carried out. The results show different protein levels among the 3 patients examined. MDM4 levels are increased in lesional skin of all three patients. However, there are big differences in non-lesional skin between the three patients **(Figure 33a)**. Densitometry analysis of MDM4 protein bands in relation to loading control confirms significantly up-regulated MDM4 expression in lesional (n=3) skin. Due to the variation in MDM4 expression of non-lesional skin, the calculated mean reveals no significant change in protein expression **(Figure 33b, c)**.

This observation holds an important message, namely, that the individual result needs to be taken into account for our complete understanding, as a look at the mean or median may give false answers.

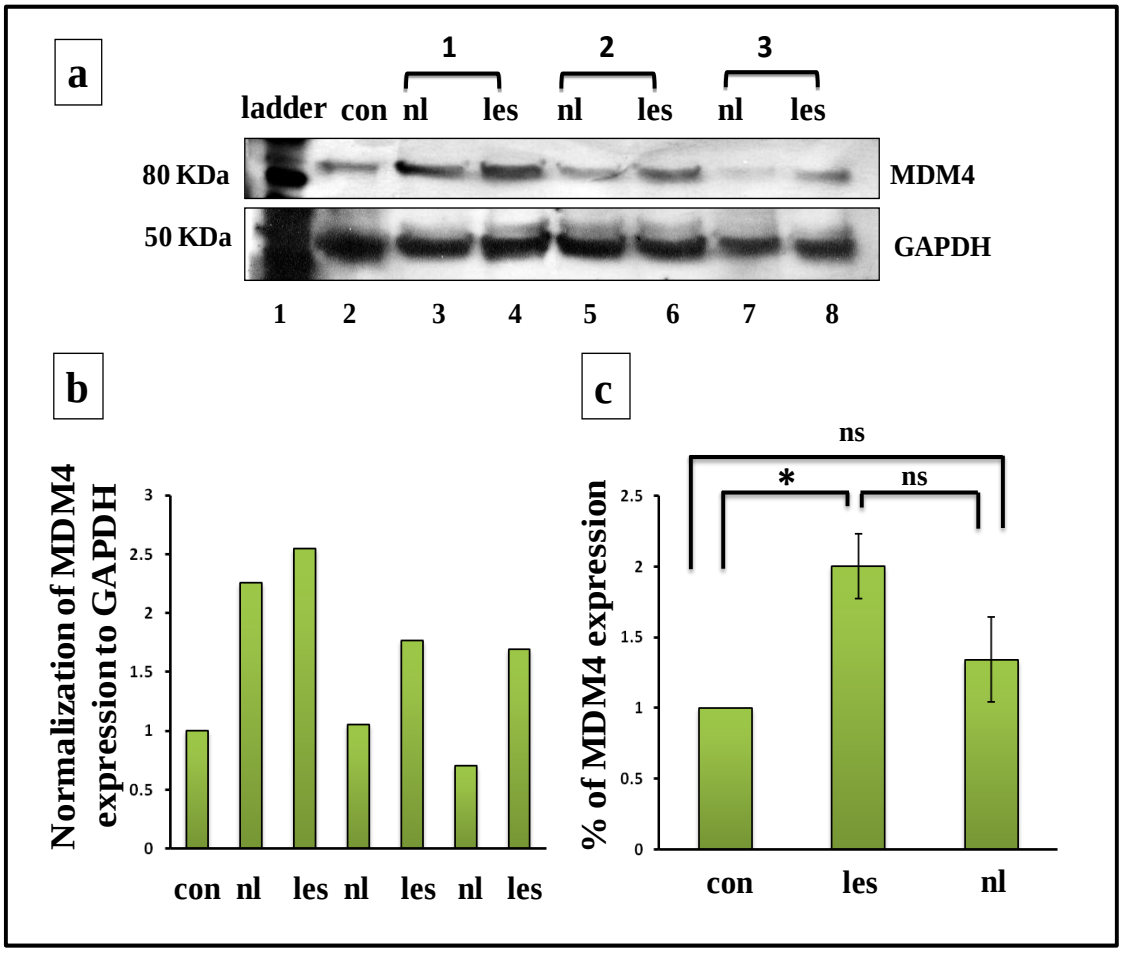

Figure 33: Up-regulated MDM4 expression in lesional epidermis of patients.

(a) Western blot. MDM4 (approximately 80KDa) shows an elevation in MDM4 expression in lesional skin of all patients compared to control. Lane 1 protein ladder, lane 2 control, lanes from 3-8 lesional and non-lesional skin cell extracts from patients (n=3). GAPDH was used as loading control.

(b) Normalization of expression to the loading control. This result shows the individual expression of the protein pointing to a wide range in between patients.

(**c) Quantification of the MDM4.** Image analysis of bands was used to quantify the expression of MDM4 protein in lesional and non-lesional skin in relation to its expression in control skin. The result reveals significantly up-regulated MDM4 expression in lesional (n=3) skin. However, the overall result in non lesional (n=3) skin of patients is not representative considering the wide range of the individual data. These data need more work. (Plots are mean ± SE) (NS $p < 0.05$, * $p > 0.05$).

4.1.6.1.3.3 Up-regulated *in situ* MDM4 expression in non-lesional melanocytes

Next we looked at MDM4 expression and localization in epidermal melanocytes using double immuno-fluorescence labelling with FITC-labelled MDM4 and TRITC-labelled NKI / beteb1. We detected positive MDM4 expression in melanocytes of non-lesional skin, while MDM4 expression is nearly absent in melanocytes of healthy controls **(Figure 34).** MDM4 expression was also not detectable by Western blot in cultured melanocytes of healthy control skin compared to the positive control **(Figure 36).** Hence, this observation supports our *in vitro* immuno-fluorescence result of MDM4 expression in normal melanocytes (**Figure 35).**

We were unable to test the expression in vitiliginous melanocytes under *in vitro* conditions due to shortage of cultured melanocytes from patients with vitiligo.

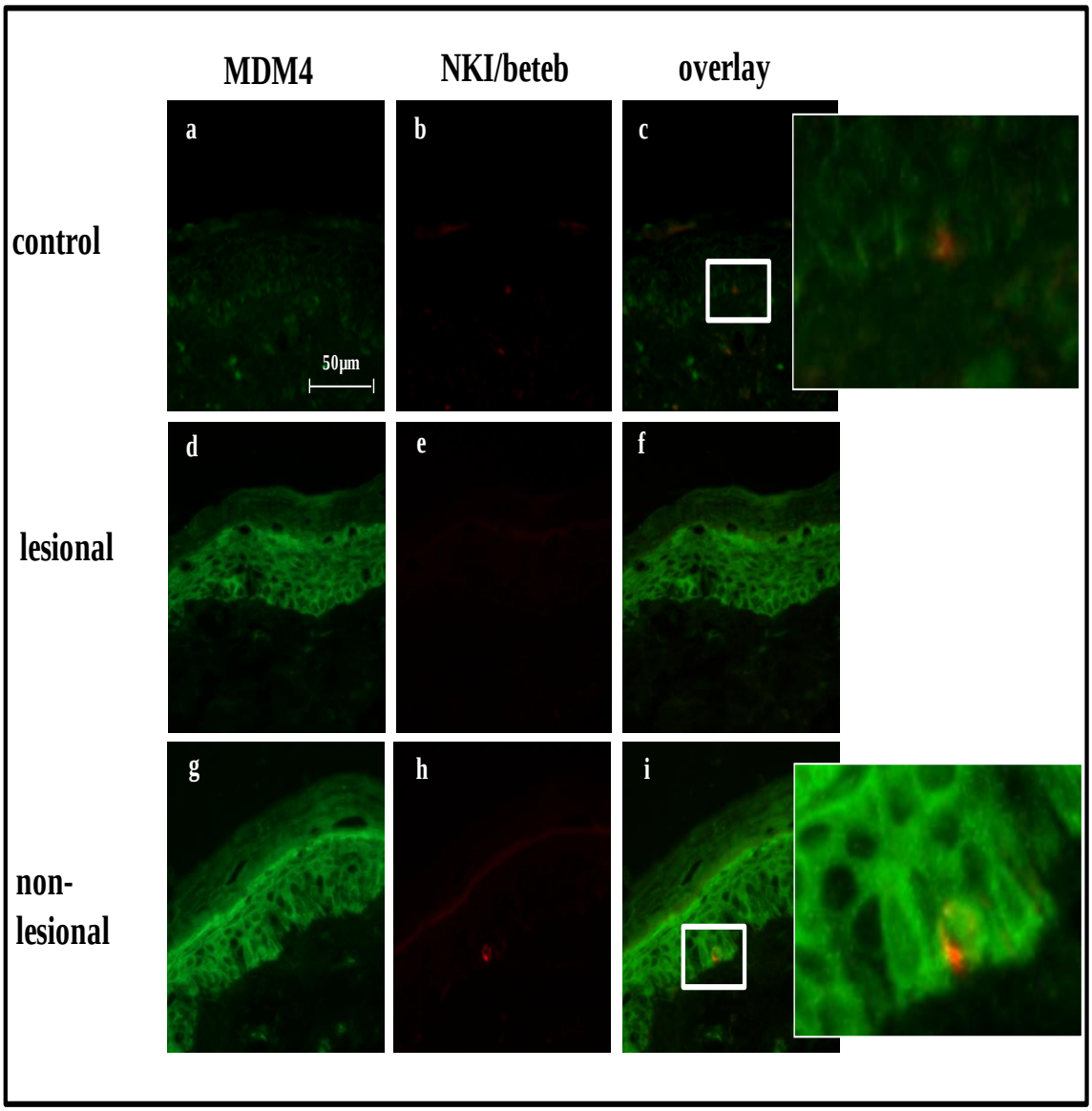

Figure 34: *In situ* MDM4 expression in epidermal melanocytes in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows in this example higher expression of MDM4 in lesional and non-lesional (d, g) skin compared to controls (a). Melanocytes are detected with TRITC-labelled NKI / beteb1. The overlay with MDM4 suggests a more pronounced MDM4 protein expression in melanocytes (orange) of non-lesional skin of a patient compared to controls (insert i). Scale bar 50µm. Magnification x 400.

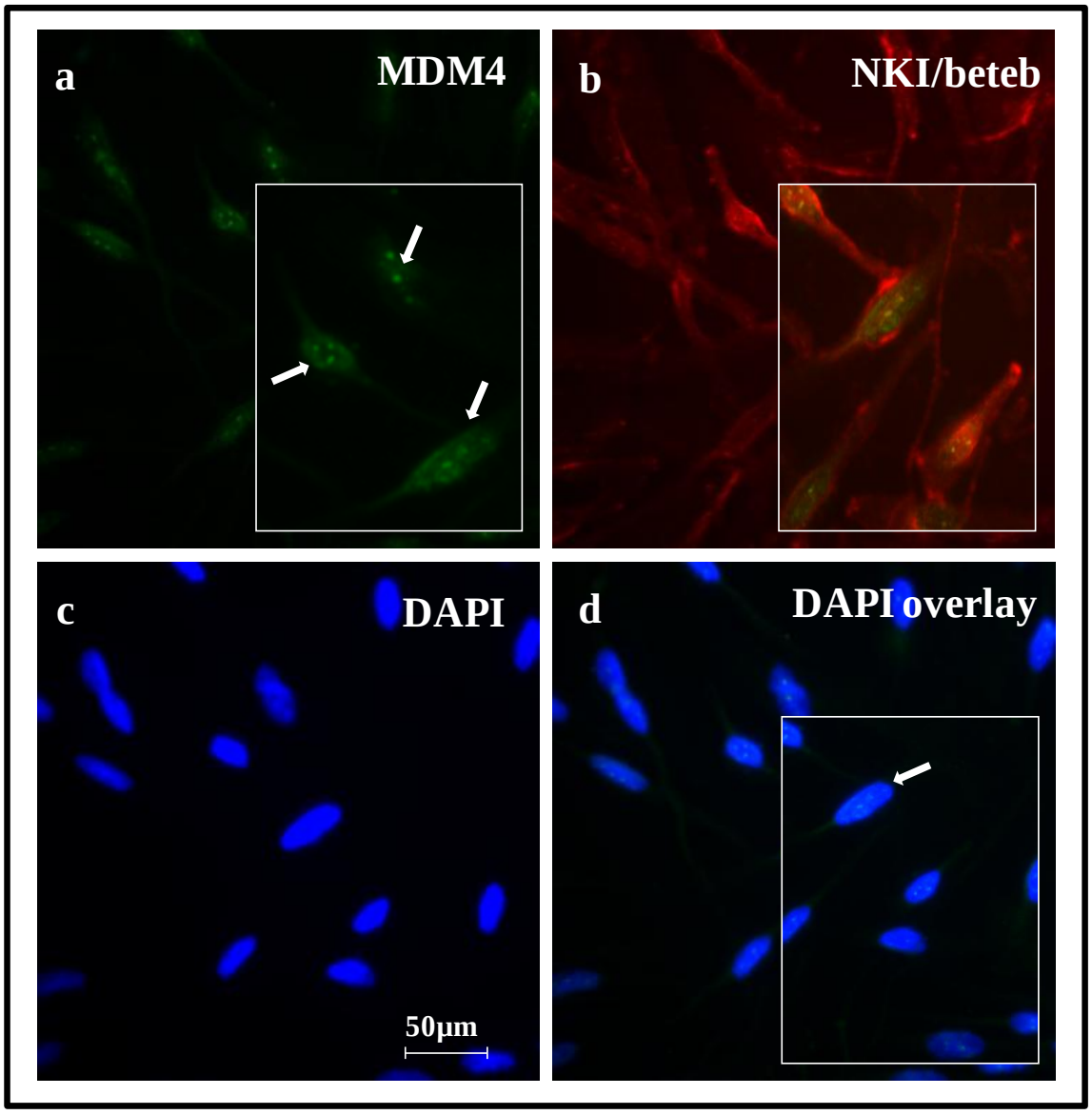

Figure 35: *In vitro* MDM4 expression is present in nucleoli in human epidermal melanocytes.

Immuno-reactivity (FITC-labelling in green) shows almost absent MDM4 in melanocytes except in some nucleoli. Overlay of NKI-beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled MDM4 shows no co-localisation of MDM4 (b and insert). Magnification x 400. Scale bar 50µm.

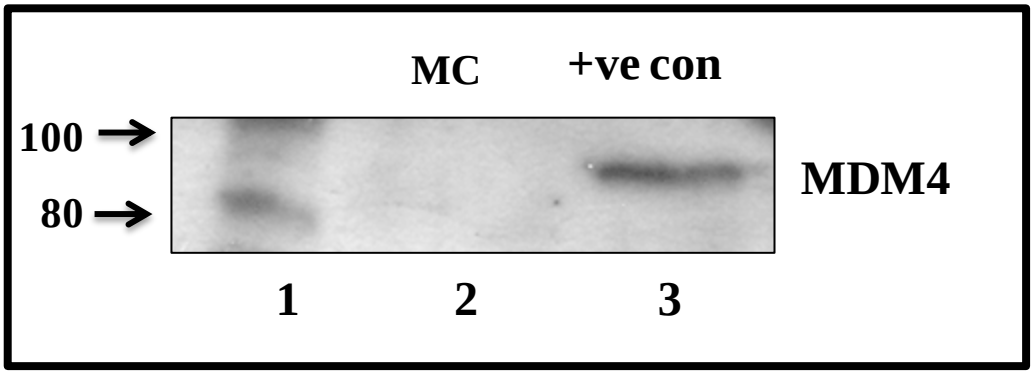

Figure 36: MDM4 is not detectable in normal human epidermal melanocytes.

Western blot shows no MDM4 band in melanocytes. Lane 1 protein ladder, lane2 normal human epidermal melanocytes and lane 3 +ve control (melanoma cells).

4.1.6.1.3.4 Significantly increased MDM4phospho expression in the entire epidermis of patients with vitiligo

Our results for MDM4phospho immuno-fluorescence labelling in vitiligo show strong expression of MDM4phospho in both lesional and non-lesional skin of patients. Overlay of FITC-labelled MDM4phospho with DAPI suggests elevated levels of MDM4phospho protein in nuclei of epidermal cells **(Figure 37)**. Image analysis of MDM4phospho expression documents significantly increased MDM4phospho protein expression in vitiligo lesional and non-lesional epidermis compared to controls **(Figure 38)**.

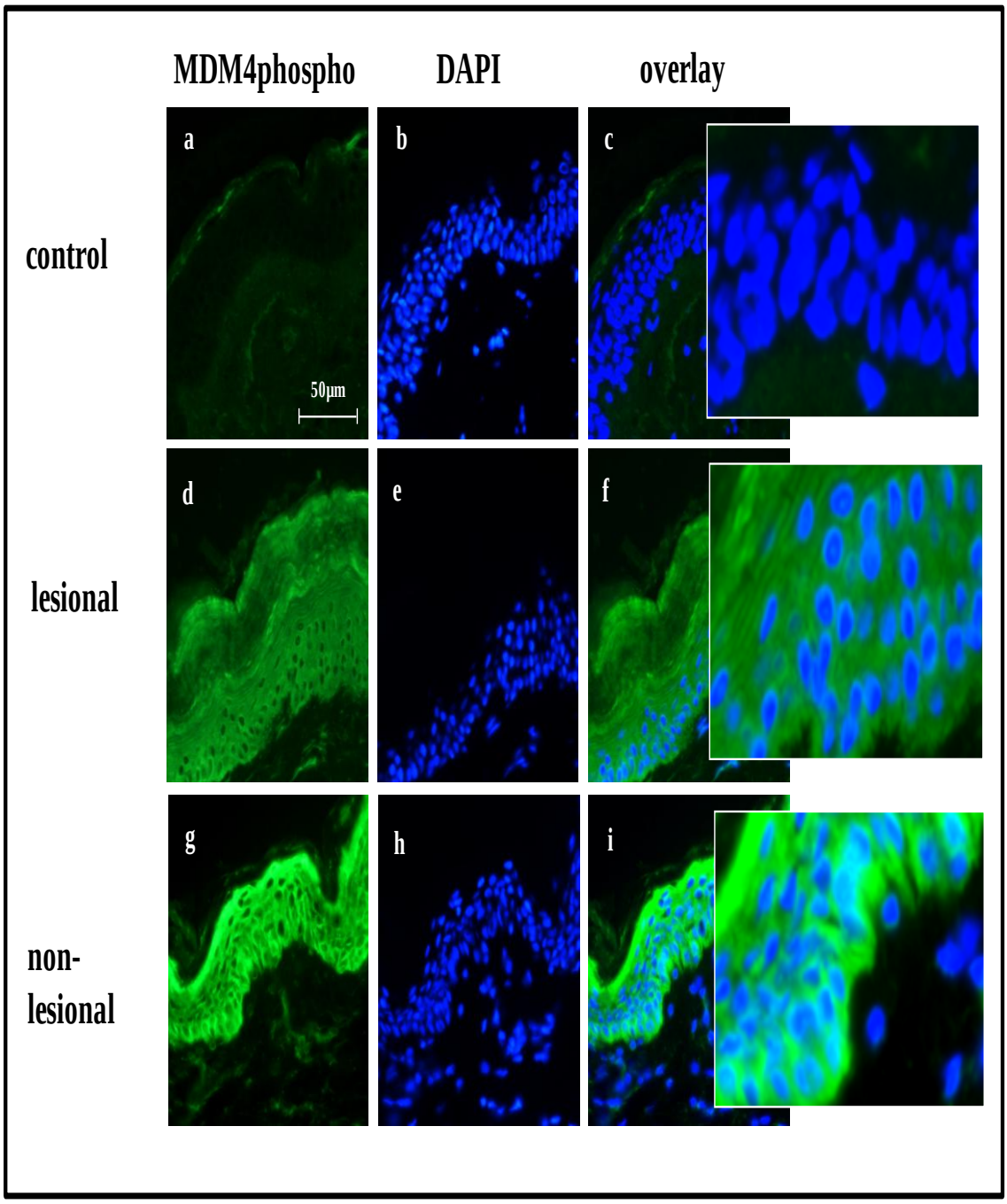

Figure 37: Increased expression of MDM4-phospho in vitiligo.

Immuno-reactivity (FITC-labelling, green) shows increased expression of MDM4phospho throughout the epidermis of both lesional (d) and non- lesional (g) skin of patients compared to controls with skin phototype III (a). Scale bar 50µm. Magnification x 400.

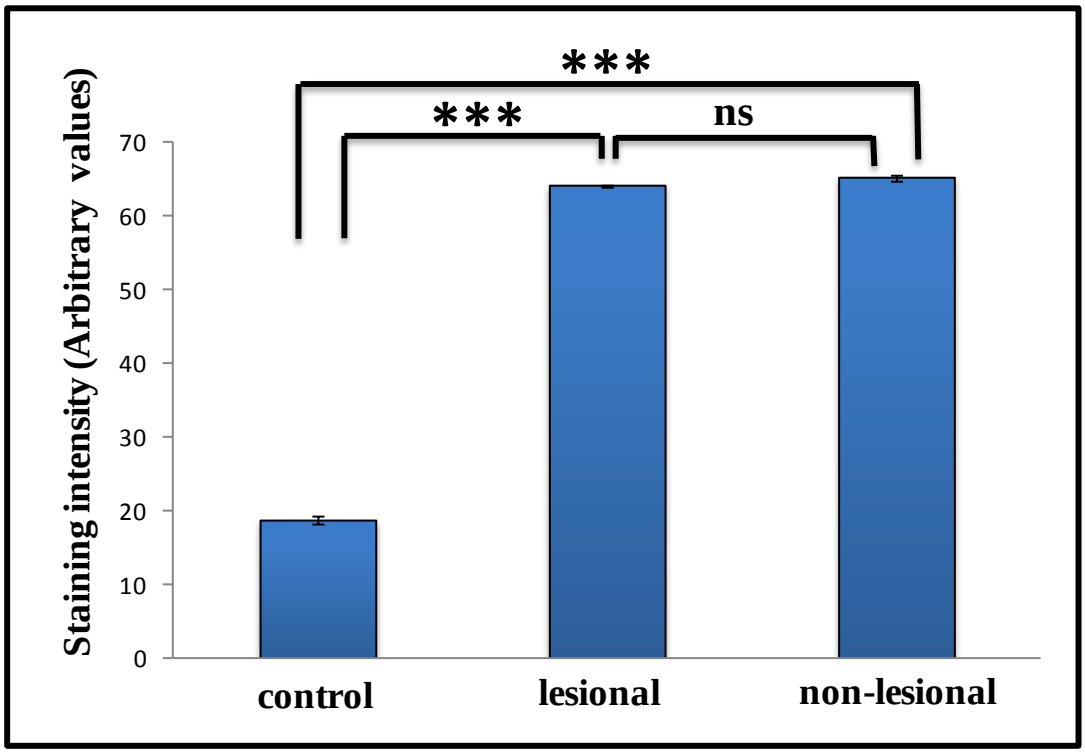

Figure 38: Significantly increased MDM4phospho expression in vitiligo.

Image analysis of the average fluorescence intensity shows significantly increased levels of MDM4phospho in the epidermis of lesional (n=45: 5 individuals, 9 pictures each) and non-lesional (n=35: 5 individuals, 7 pictures each) skin compared to controls (n=24: 3 individuals, 8 pictures each) (Plots are mean ± SE) (*** $p<0.001$, NS $p>0.05$

4.1.6.1.3.5 MDM4phospho expression levels differ between patients with vitiligo

Western blot of MDM4phospho protein expression was utilised for protein quantification. The results show big variations between the patients, using normalization of MDM4phospho levels to the loading control GAPDH in lesional and non-lesional skin compared to controls **(Figure 39 a, b)**. Image analysis confirms the results **(Figure 39 c)**. This result warrants further investigation as the data point to big differences in patients. Moreover, this result points to the limitation of statistics, because the overall result looses the increased levels shown in one patient.

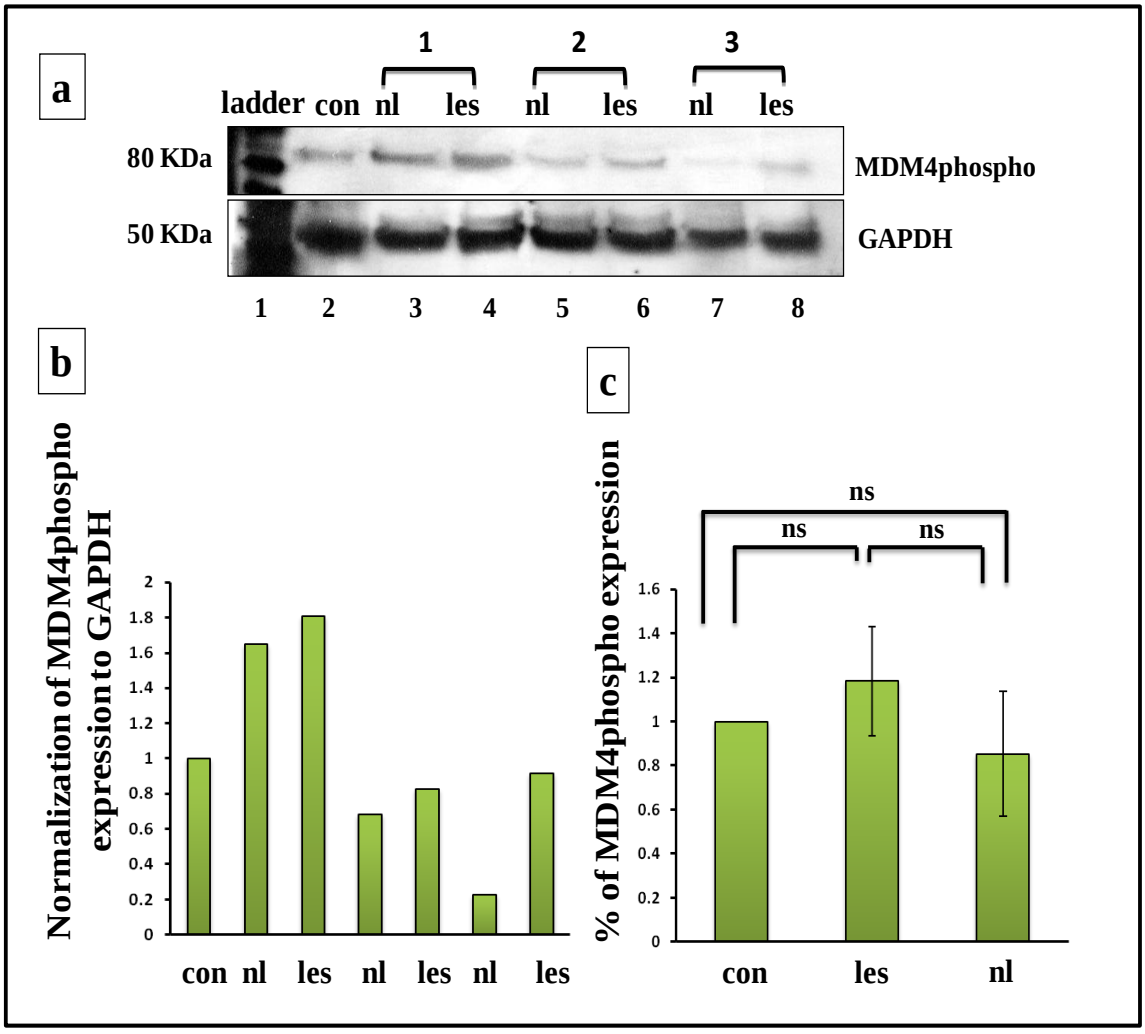

Figure 39: Epidermal MDM4phospho expression differs in patients.

(a) Western blot. MDM4phospho shows similar expression of MDM4phospho protein (approximately 80 KDa) in lesional and non-lesional skin of two patients with vitiligo compared to healthy control, while one patient shows significantly higher expression. Lane1 protein ladder, lane 2 control, lanes from 3-8 lesional and non-lesional epidermal suction blister tissue extracts from 3 vitiligo patients. GAPDH was used as loading control.

(b) Normalization of expression to the loading control reveals different individual expression.

(**c) Quantification of the MDM4phospho.** Image analysis of bands used to quantify the expression of MDM4phospho protein in lesional and non-lesional skin in relation to its expression in control skin. The overall result reveals no significant change in the levels of MDM4phospho expression in both lesional (n=3) and non-lesional (n=3) skin of patients with vitiligo compared to normal healthy control (n=1). (Plots are mean ± SE) (NS $p>0.05$).

The elevated MDM4phospho in only one of three patients` samples together with up-regulated MDM4 expression in lesional skin of the same patients as well as the up-regulated p21 levels in their skin got our attention for further evaluation. Therefore we looked at the MDM4/MDM4phospho ratio in these samples to get a better understanding about MDM4 and its possible role in vitiligo. Analyses of MDM4 / MDM4phospho ratio in these patients exhibit higher MDM4 levels than the corresponding phosphorylated protein in individual patients compared to healthy controls. This result suggests that not all MDM4 protein is phosphorylated in skin of these patients and phosphorylation levels can greatly vary between patients (**Table 5).**

Protein levels	**control**	**Pat 1 non-les**	**les**	**Pat 2 non-les**	**les**	**Pat 3 non-les**	**les**
MDM4	**1**	**2.26**	**2.55**	**1.06**	**1.77**	**0.71**	**1.69**
MDM4phospho	**1**	**1.651**	**1.81**	**0.68**	**0.83**	**0.23**	**0.91**
MDM4/ MDM4phospho ratio	**1**	**1.37**	**1.41**	**1.54**	**2.14**	**3.10**	**1.85**
calculated un-phosphorylated MDM4	**0**	**0.61**	**0.74**	**0.37**	**0.94**	**0.48**	**0.78**

Table 5

MDM4-MDM4phospho ratio in individual vitiligo patients (n=3).

4.1.6.1.3.6 MDM4phospho is highly expressed in non-lesional melanocytes in vitiligo

To investigate the expression of MDM4phospo protein in epidermal melanocytes of non-lesional skin of patients, we again utilised double immuno-fluorescence labelling. Overlay of FITC-labelled MDM4phospho and TRITC-labelled NKI/beteb1 suggests higher MDM4phospho expression in melanocytes of non-lesional skin compared to almost absent expression in melanocytes of healthy control skin (**Figure 40).** MDM4phospho expression in normal melanocytes under *in vitro* conditions confirms almost absence in these cells. Very little MDM4phospho protein expression is present in nucleoli and perinuclear cytoplasm **(Figure 41).** To quantify levels of MDM4phospho expression in normal human epidermal melanocytes, Western blot was utilised. MDM4phospho expression was not detected in our samples compared to the positive control **(Figure 42)**, supporting in turn the *in vitro and in situ* results. However, whether, there are some patients with higher expression still needs to be shown.

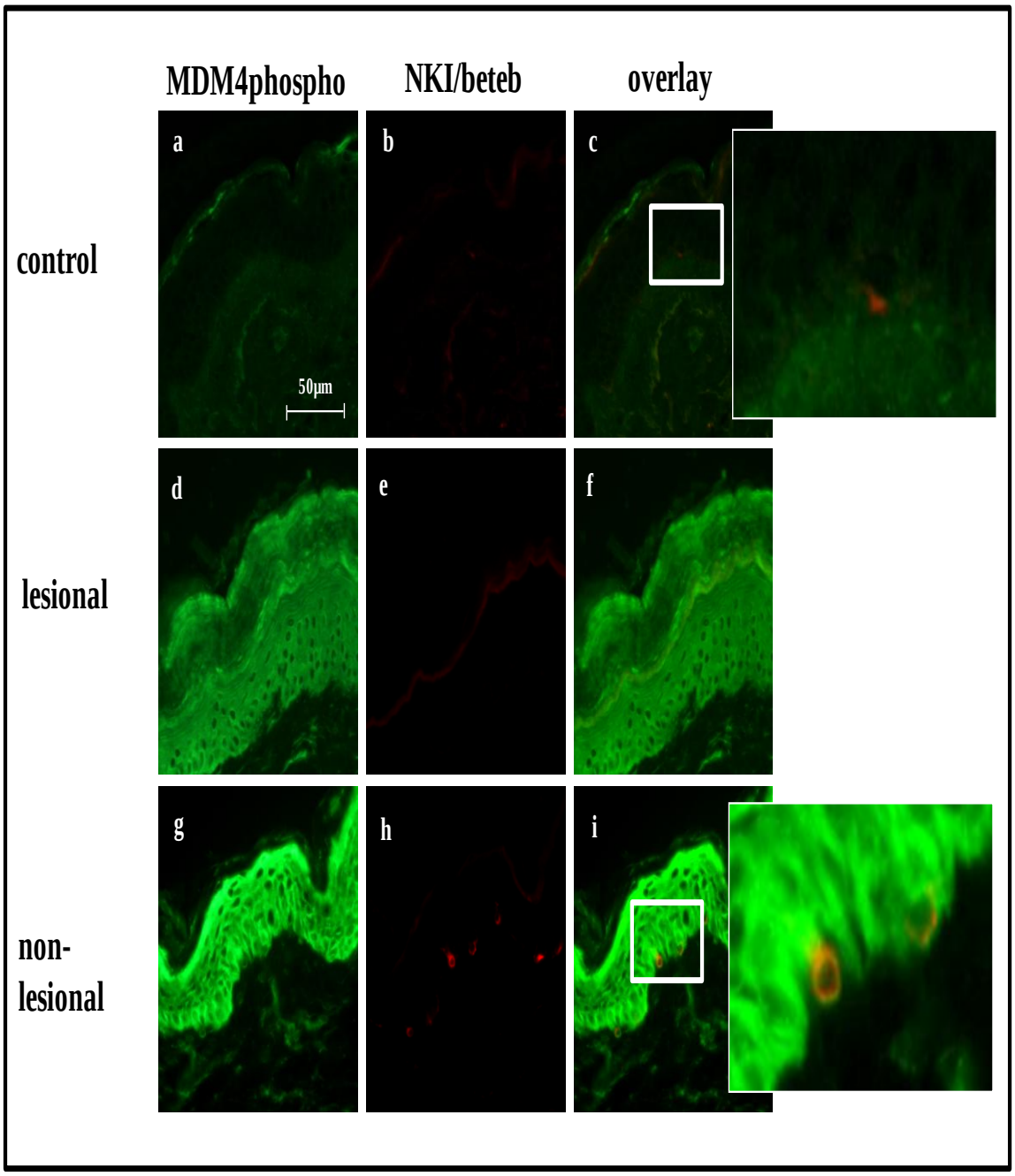

Figure 40: High *in situ* expression of MDM4phospho in melanocytes of patients.

Immuno-reactivity staining (FITC-labelling green) shows considerable increased expression of MDM4phospho throughout the entire epidermis of both lesional (d) and non-lesional (g) skin in this example of vitiligo compared to control with skin phototype III. Melanocytes of non-lesional skin (i) show stronger MDM4phospho expression compared to normal skin after overlay with NKI/beteb1 (c). Scale bar 50µm. Magnification x 400.

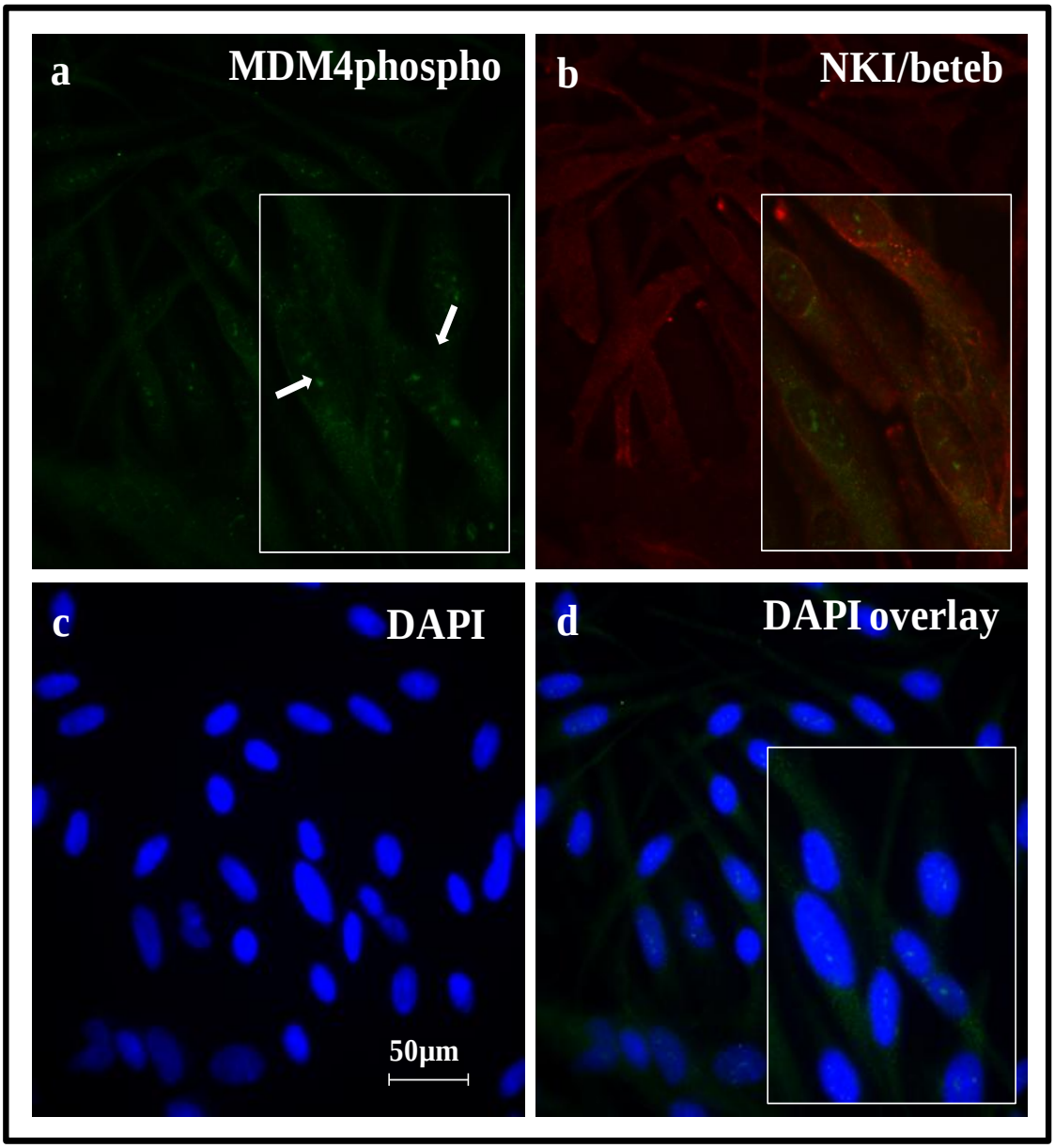

Figure 41: Absent *in vitro* expression of MDM4phospho in human epidermal melanocytes.

Immuno-reactivity (FITC-labelling in green) shows background MDM4phospho expression in normal melanocytes. Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled MDM4phospho shows no co-localisation of MDM4phospho in melanosomes (b and insert). Magnification x 400. Scale bar 50µm.

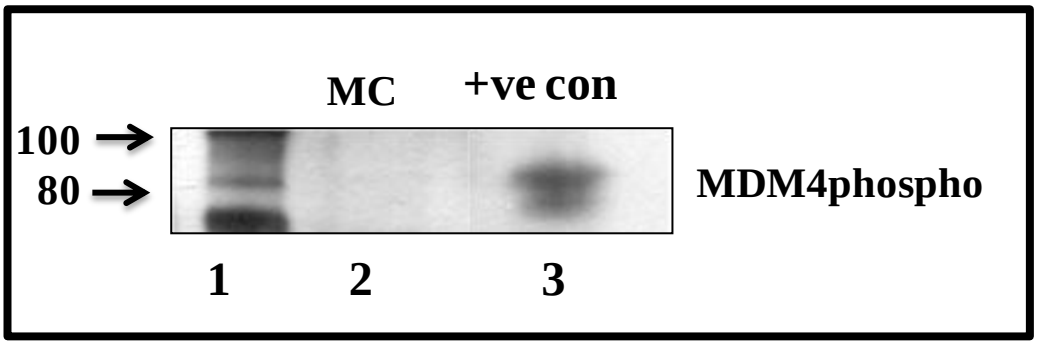

Figure 42: MDM4phospho is not detectable in epidermal melanocytes.

Western blot shows no MDM4phospho protein band in human epidermal melanocytes. Lane 1 protein ladder, lane 2 normal human epidermal melanocytes and lane 3 +ve control (melanoma cells).

4.2 Investigation of the SPARC- p53 axis in vitiligo

4.2.1 Significantly up-regulated SPARC-expression in the entire epidermis of patients with vitiligo - a novel observation

Briefly, SPARC is an important regulator of p53. Recently it was shown that depletion of SPARC in melanoma cells results in activation of p53 and induction of p21 leading in turn to G2/M cell cycle arrest and tumour growth inhibition (Fenouille et al., 2011). We therefore asked the question, whether SPARC could be a possible player in the up-regulation of p53 in vitiligo.

Our results show strong *in situ* expression of SPARC protein throughout the entire epidermis in lesional (d) and non-lesional (g) skin of patients compared to controls (a) **(Figure 43).** Image analysis of SPARC proves significantly elevated protein expression in lesional ($p<0.001$, mean ± SE) and non-lesional ($0.01>p>0.001$±SE) epidermis compared to controls **(Figure 44)**.

To our knowledge this is the first report on high expression of SPARC in the entire epidermis of patients with vitiligo, together with persistent up-regulated functioning $p53^{w/w}$ and increased p21 expression in the presence of 10^{-3}M H_2O_2 confirmed by corresponding low catalase expression **(Figures 14, 20, 24)** and *in vivo* FT-Raman spectroscopy (**Figure 18).**

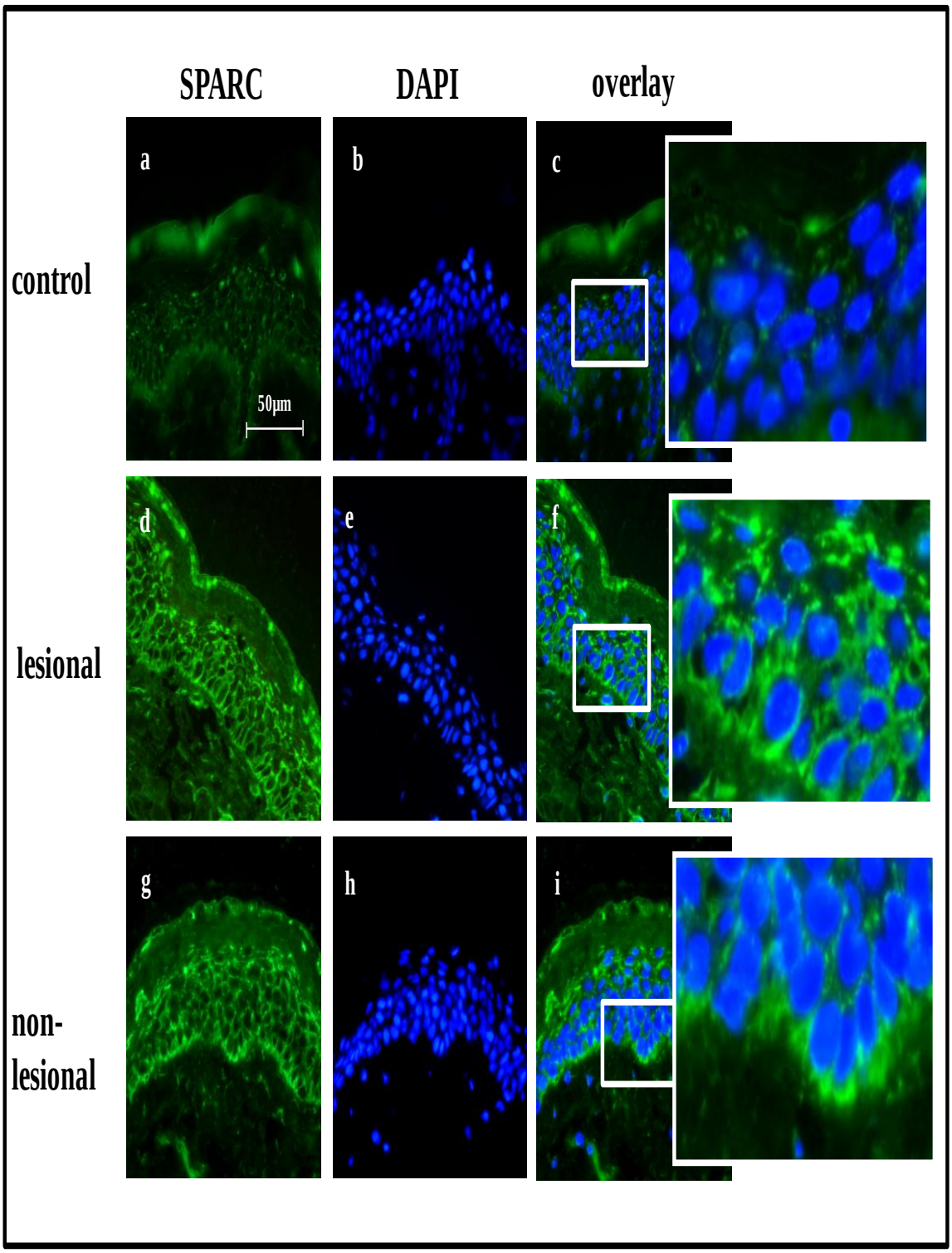

Figure 43: High expression of SPARC in vitiligo.

Immuno-reactivity (FITC- labelling, green) shows an increased expression of SPARC in lesional (g) and non-lesional (d) skin of vitiligo patient compared to healthy control skin phototype III (a). Scale bar 50µm. Magnification x 400.

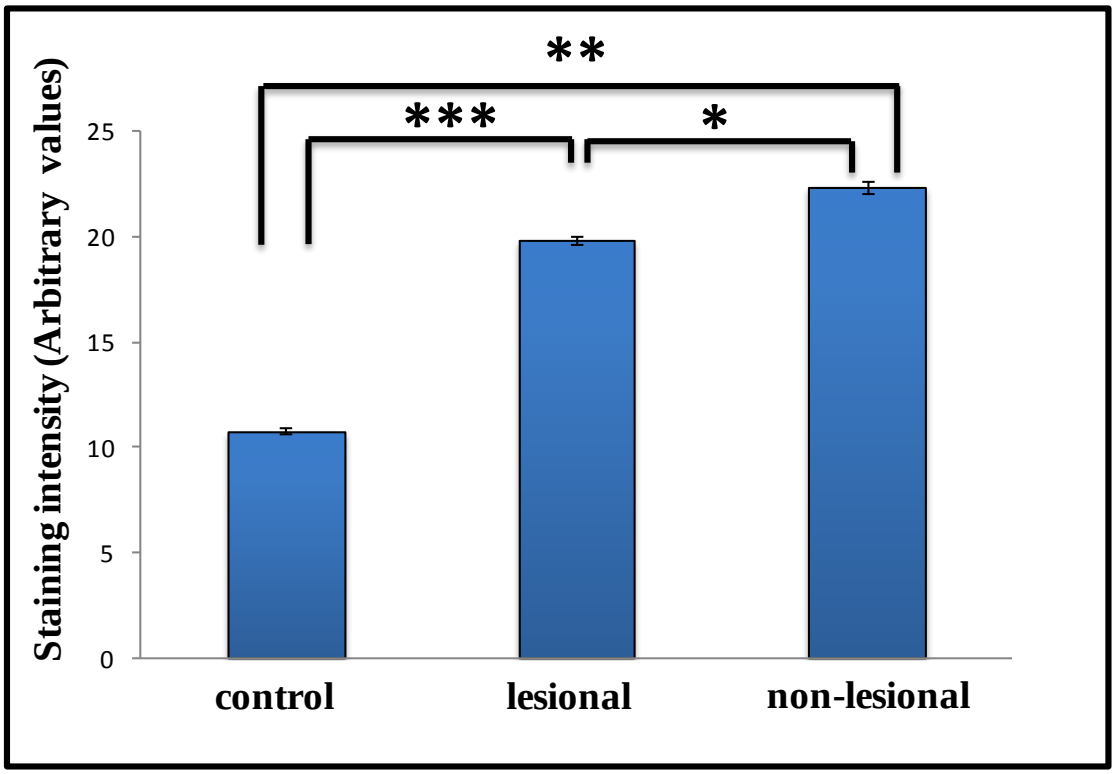

Figure 44: Significantly up-regulated expression of SPARC in vitiligo.

Image analysis of the average fluorescence intensity supports significantly increased levels of SPARC in both vitiligo lesional (n=42: 7 individuals, 6 pictures each) and non-lesional (n=42: 7 individuals, 6 pictures each) skin compared to controls (n=28: 4 individuals, 7 pictures each). (Plots are mean ± SE) (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

4.2.2 More support for high epidermal SPARC protein levels in vitiligo

For further support of our *in situ* SPARC protein expression, we utilised Western blot. The results show up-regulated SPARC expression at the expected size of approximately 40 kDa in lesional (n=3) and non-lesional epidermal cell extracts (n=3) compared to skin of healthy controls **(Figure 45a)**. Image analysis of SPARC protein bands in relation to loading control protein (GAPDH) reveals significantly increased SPARC expression in all samples **(Figure 45b, c)**.

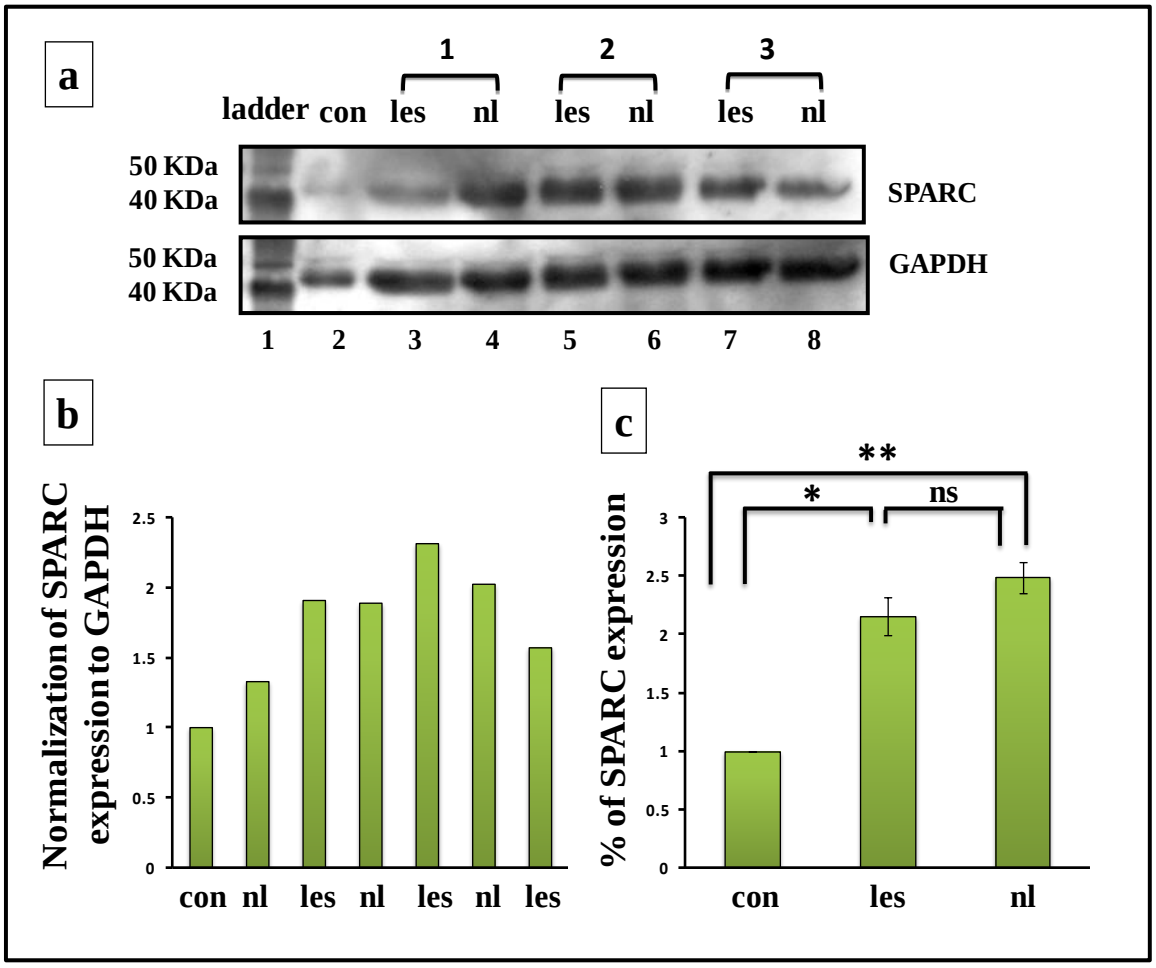

Figure 45: Significantly up-regulated epidermal SPARC expression in vitiligo.

(a) Western blot. SPARC protein is seen at ~43KDa with elevated expression in both lesional and non-lesional skin of patients compared to healthy controls. Lane 1 protein ladder, lane 2 is control, lanes 3-8 lesional and non-lesional skin cell extracts from 3 vitiligo patients. GAPDH was used as loading control.

(b) Normalization of SPARC expression to the loading control. GAPDH was used to evaluate the expression of the individual protein levels. The results confirm increased levels in all patients examined.

(c) Quantification of SPARC bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals up-regulated expression in both lesional (n=3) and non- lesional (n=3) skin of patients with vitiligo compared to control (n=1). (Plots are mean ± SE) (NS $p > 0.05$, * $p<0.05$, ** $p<0.01$).

4.2.3 H_2O_2 does not affect the antibody binding site of SPARC

Next we needed to ensure that the epitope for the antibody used for immuno-fluorescence and Western blot is not affected by mM levels of H_2O_2. Therefore we performed dot blot analysis with recombinant SPARC protein after incubation with different mM concentrations of H_2O_2. Our results demonstrated no differences in reaction of the antibody with SPARC protein in the presence of different H_2O_2 concentrations. Based on this result, we can conclude that the epitope for antibody binding is not affected by oxidation **(Figure 46).**

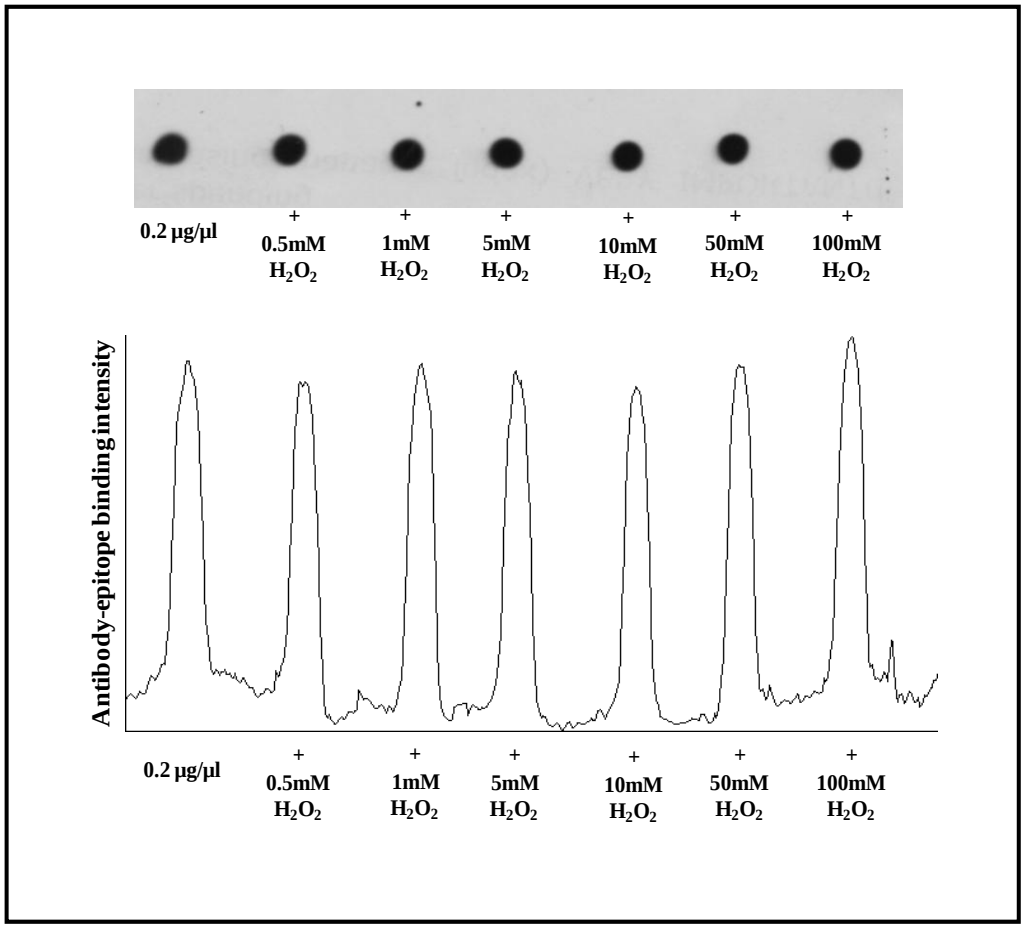

Figure 46: **The effect of H_2O_2 on SPARC protein**.

Dot blot shows no effect of H_2O_2 on the immuno reactivity of the SPARC epitope.

4.2.4 SPARC expression is increased in non-lesional melanocytes

We next looked at *in situ* and *in vitro* SPARC expression and localization in melanocytes using double immuno-fluorescence labelling with FITC-labelled SPARC and TRITC- labelled NKI / beteb1. The *in situ* results identify strong SPARC expression (in yellow) after overlay of NKI / beteb1 and SPARC in non-lesional melanocytes, while SPARC is absent in melanocytes of control skin **(Figure 47).** Moreover, our *in vitro* results of SPARC expression in normal epidermal melanocytes show strong localisation of SPARC protein in nuclear envelop, nucleoli and cytoplasm but without overlap with melanosomes **(Figure 48).** For quantification of SPARC expression in normal human epidermal melanocytes, Western blot was utilised. The results confirmed the *in situ* expression of SPARC protein in normal melanocytes compared with the band of a positive control **(Figure 49).**

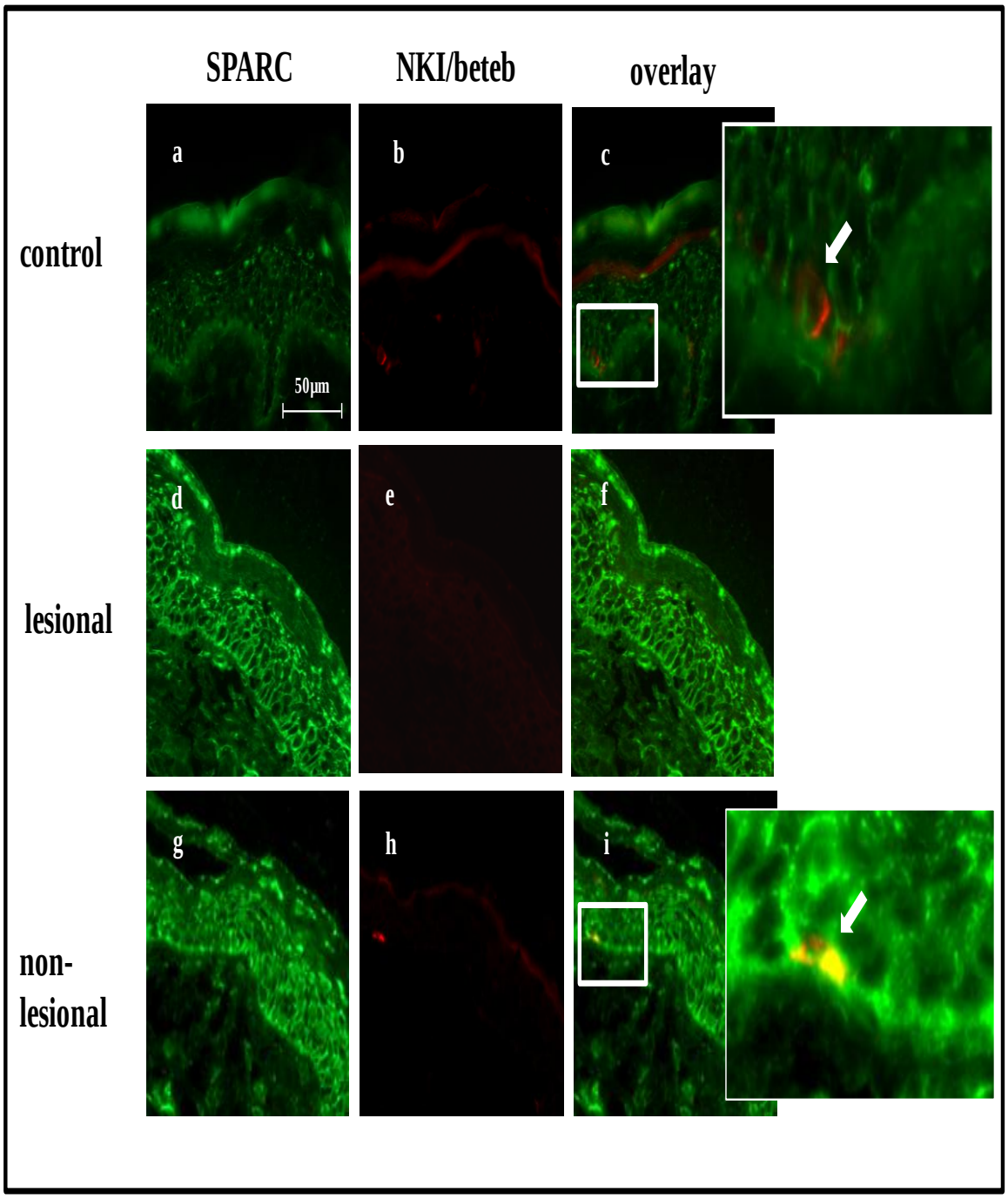

Figure 47: Increased SPARC expression in epidermal melanocytes of non-lesional vitiligo.

Immuno-reactivity (FITC-labelling, green) shows strong expression of SPARC in lesional and non-lesional (i) epidermis of patients, while SPARC cannot be detected in healthy controls (c) with skin phototype III. Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled SPARC identifies SPARC expression (yellow) in vitiligo (f; i) and insert, while SPARC is absent in normal control melanocytes (c) and insert. Magnification x 400. Scale bar 50µm.

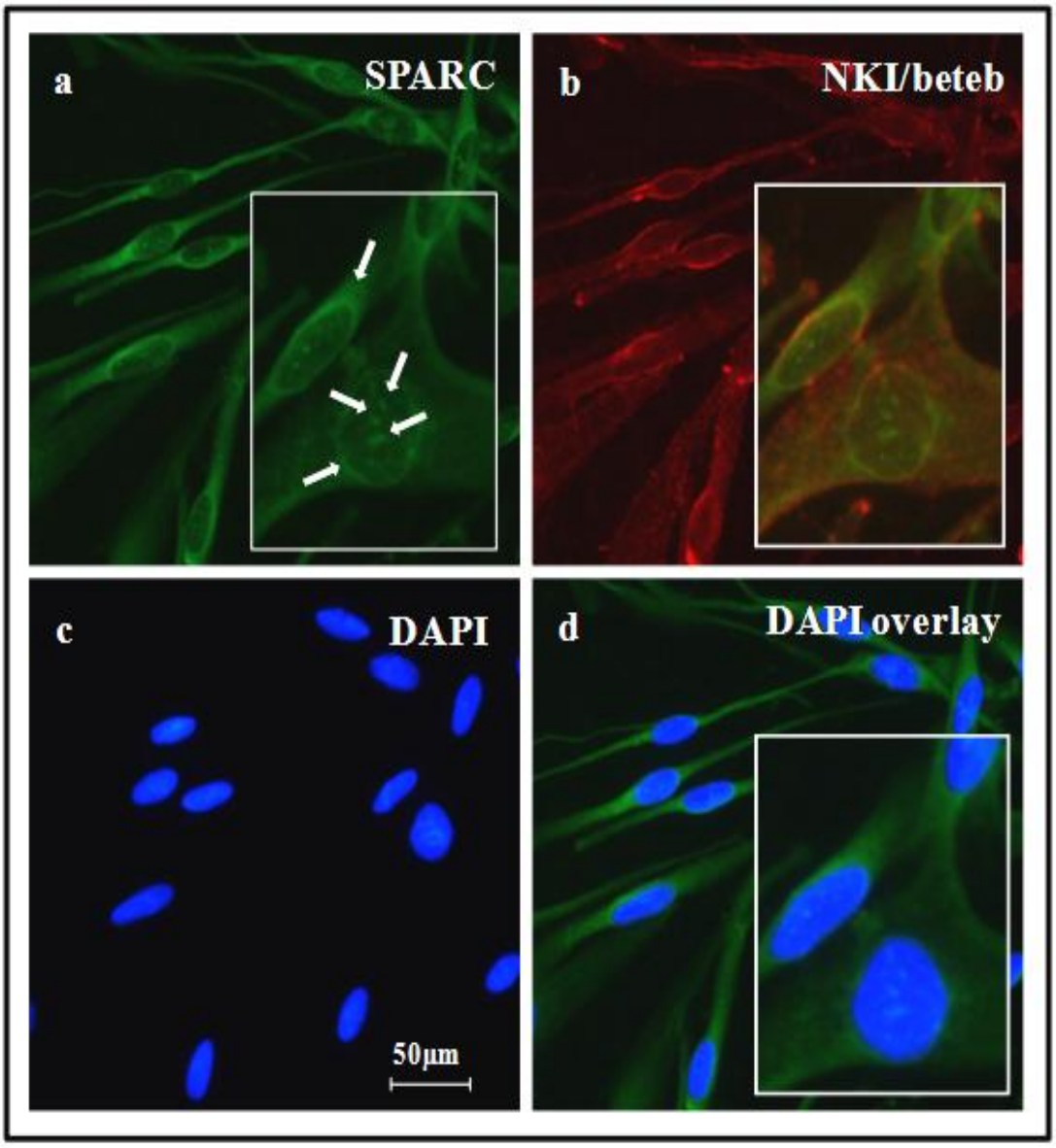

Figure 48: *In vitro* expression of SPARC in human epidermal melanocytes.

Immuno-reactivity (FITC-labelling, green) shows that SPARC is expressed in melanocytes under *in vitro* conditions. Patterns of expression indicate that SPARC is present in peri-nuclear cytoplasm and possibly in nucleoli (a insert). Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled SPARC shows no co-localisation of SPARC in melanosomes (b and insert). Magnification x 400. Scale bar 50µm.

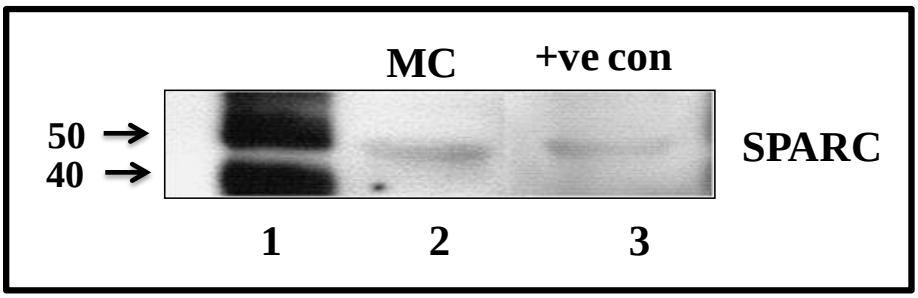

Figure 49: SPARC is present in human epidermal melanocytes.

Western blot supports the presence of SPARC in human epidermal melanocytes. The band observed at approximately 40 kDa is in agreement with the size of SPARC. Lane 1 protein ladder, lane 2 normal human epidermal melanocytes and lane 3 +ve control (Santa Cruz Biotechnology Inc, sc-3811).

4.2.5 Does elevated nitric oxide (NO) affect epidermal SPARC in vitiligo?

One question to be answered was, whether peroxynitrite ($ONOO^-$) could affect SPARC functionality in vitiligo.

Briefly, NO is synthesised from the terminal guanido nitrogen atom of L-arginine by the NADPH- dependent enzymes i.e.NO- synthases (NOS). Rapid reaction of NO with $O_2^{\cdot -}$ generates peroxinitrite anion ($ONOO^-$) (Burney et al., 1999) which in turn can nitrate the phenol ring of L-tyrosine in cellular proteins (Mannick and Schonhoff, 2004).

Previously it was shown that $ONOO^-$ is highly expressed in skin of patients with vitiligo (Salem et al., 2009).

Hence, it was tempting to evaluate the effect of SPARC on p53 regulation in the presence of this radical, using 5-nitro- tyrosine expression as the foot print for $ONOO^-$.

4.2.5.1 High expression of 5-nitro-tyrosine in the epidermis of patients with vitiligo confirms the presence of $ONOO^-$

In order to study possible nitration of SPARC in vitiligo, we evaluated the presence of $ONOO^-$ via immuno-fluorescence staining using TRITC-labelled 5-nitro-tyrosine. Our *in situ* results confirmed the presence of 5-nitro-tyrosine throughout the entire epidermal compartment in both lesional and non-lesionl skin of patients **(Figure 50).** From this *in situ* result we can conclude that $ONOO^-$ is indeed present in our patients. Moreover, these results are in agreement with our previous findings. (Salem et al 2009).

Overlay with DAPI indicates the presence of $ONOO^-$ also in the nucleus. Image analysis of 5-nitro-tyrosine expression reveals significantly up-regulated $ONOO^-$ levels in lesional and non-lesional epidermis compared to controls **(Figure 51)**.

4.2.5.2 In vitiligo SPARC is nitrated throughout the entire epidermis

Double immuno-fluorescence labelling of FITC-labelled SPARC and TRITC-labelled 5-nitro-tyrosine proves for the first time high expression of nitrated SPARC in lesional and non-lesional skin of patients with vitiligo. This elevated expression of nitrated SPARC is more pronounced in cellular walls in addition to some nuclei **(Figure 50)**.

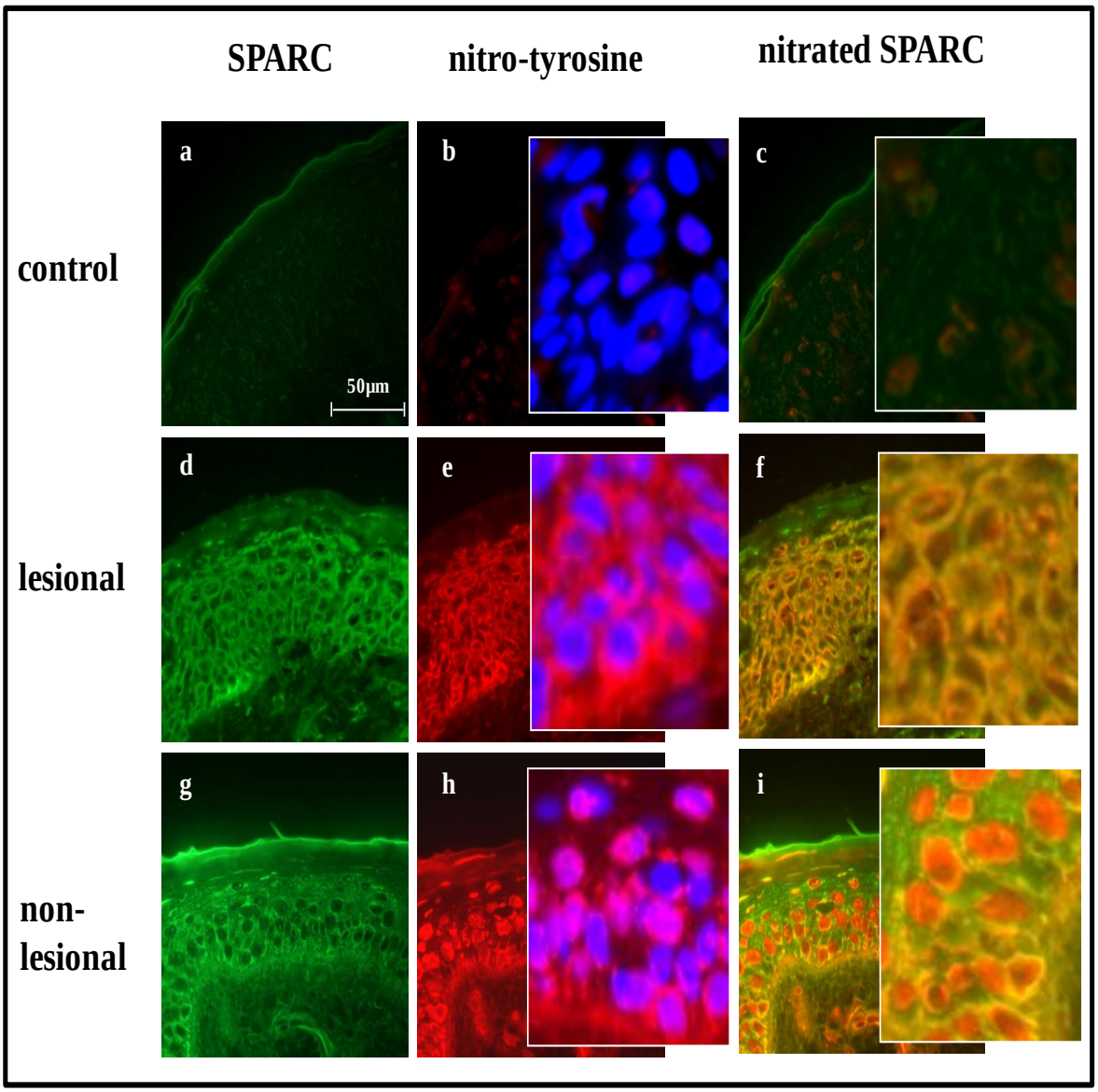

Figure 50: Increased *in situ* expression of nitrated SPARC in vitiligo.

Double immuno-fluorescence of TRITC-labelled 5-nitro-tyrosine and FITC-labelled SPARC shows strong expression of nitrated L-tyrosine in vitiligo lesional (e) and non-lesional (h) skin. Overlay of nitrated tyrosine with DAPI suggests its localization in the nuclei (purple) throughout the epidermis of vitiligo lesional and non- lesional skin (e,h inserts). It also identifies the presence of nitrated SPARC in the entire skin of patients with witiligo. Nitrated SPARC is present in the cell wall and in some nuclei. It is absent in healthy controls (c) (skin phototype III, Fitzpatrick classification). Scale bar 50µm. Magnification x 400.

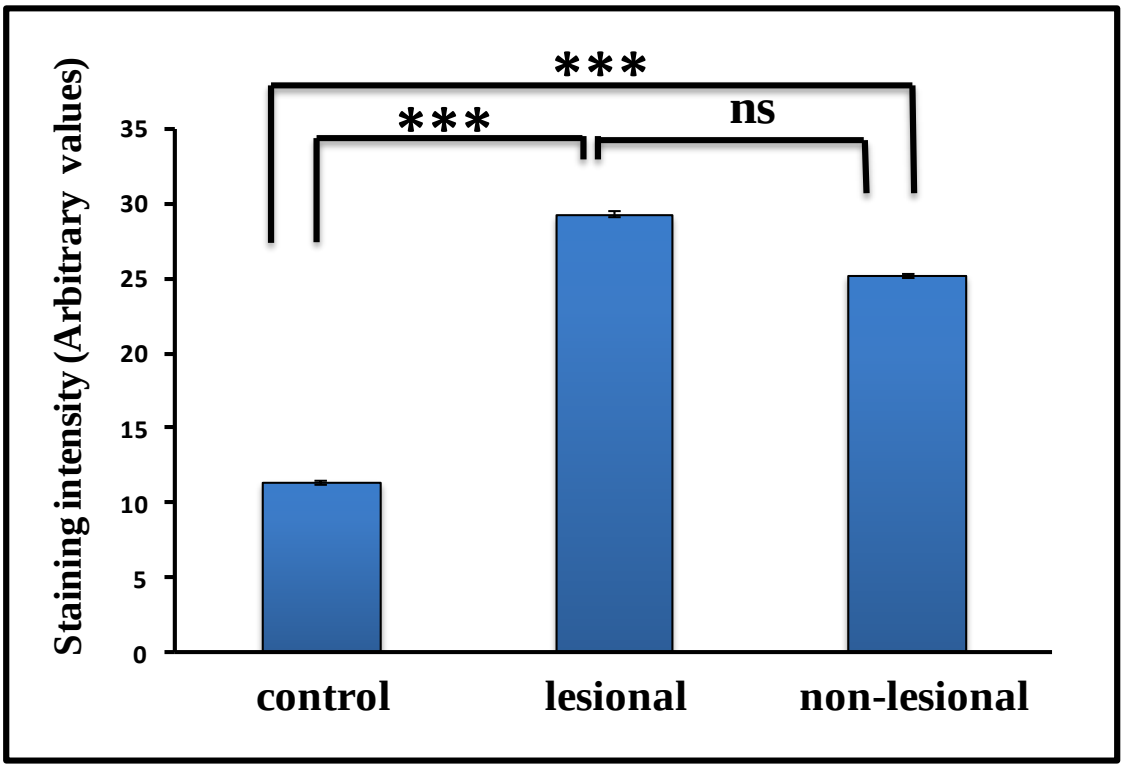

Figure 51: Significantly up-regulated nitration in vitiligo.

Image analysis of the average fluorescence intensity shows significantly increased levels of 5-nitro-tyrosine in vitiligo lesional (n=24: 4 individuals, 6 pictures each) and non-lesional (n=24: 4 individuals, 6 pictures each) skin compared to controls (n=20: 2 individuals, 10 pictures each). (Plots are mean ± SE) (*** $p<0.001$, NS $p>0.05$).

4.2.5.3 Weak SPARC *in vitro* nitration in normal epidermal melanocytes

Under *in vitro* conditions SPARC and 5-nitro-tyrosine are expressed in normal human melanocytes with weak nuclear co-localization (**Figure 52).**

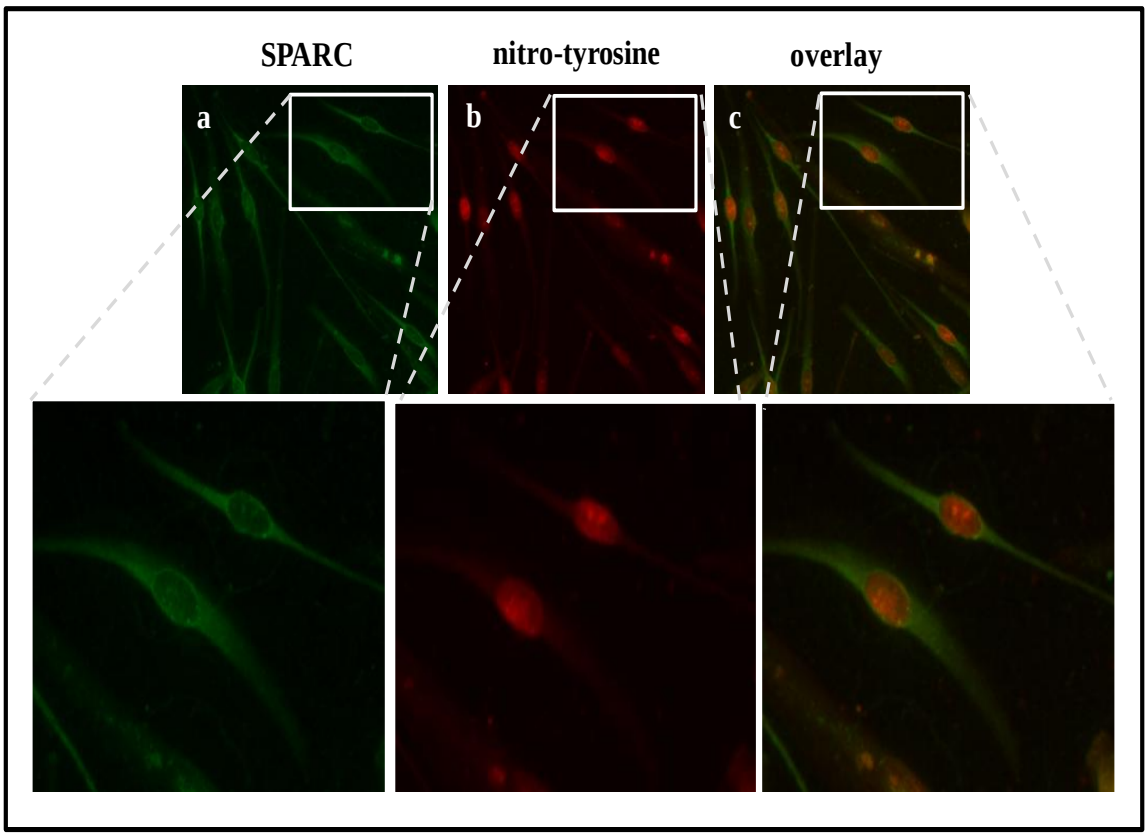

Figure 52: *In vitro* nitration of SPARC in nuclei of normal melanocytes.

Immuno-fluorescence of TRITC-labelled 5-nitro-tyrosine and FITC-labelled SPARC shows almost absent nitration of SPARC by the overlay of both chromophores. However, melanocytes nuclei show weak co-expression (a, b, c insert). Scale bar 50µm. Magnification x 400.

4.2.5.4 Computer modelling supports deactivation of SPARC-functionality by H_2O_2-mediated oxidation and $ONOO^-$- mediated nitration

Mature SPARC is 286 amino acids in length, and consists of 3 domains: an acidic flexible N-terminal domain (1-52), a Follistatin (FS) like domain which contains a single (52-137) glycosylation site (Maurer et al., 1995), and a C-terminal EC domain. X-ray crystallographic studies have revealed much of the structure and function of SPARC. The structure of the isolated EC domain was solved first, followed by the combined structures of the FS and EC domains (Hohenester et al., 1996, 1997). The structure of the C-terminal domain has not been solved yet, as it appears to be random. SPARC function is known to be calcium dependent. The main site for calcium binding is in the EC domain, which contains two high affinity EF-hands binding sites for calcium (Hohenester et al., 1996, 1997). It is also thought that the C-terminus, which is rich in glutamic acids may bind calcium and hydroxyapatite, but with lower affinity (Maurer *et al* 1992). These EF-hands are unusual; the first contains an insertion of amino acid, which is accommodated by a cis - peptide bond and the substitute of a backbone carbonyl for the more usual side chain. The second EF-hand is stabilised by a disulfide bond. Subsequent structural studies, involving a recombinant mutant as well as site directed mutagenesis, investigated the binding of SPARC to collagen. It is known that SPARC can be activated by proteolytic cleavage by either metalloproteinases or other endogenous proteinases (Sasaki et al., 1997). These studies implicated several residues critical or significant to binding. Moreover, they revealed that proteolytic cleavage of a specific α-helix exposes some of these residues which are buried, making in turn collagen binding easier and increasing affinity. The initial studies suggested several hydrophobic residues important for binding (Sasaki et al., 1998).

4.2.5.4.1 H_2O_2-mediated oxidation and $ONOO^-$-mediated nitration alter calcium binding of native SPARC

As said above, there are 2 calcium binding sites in SPARC, situated in the EC domain **(Figures 53and 55).** Both sites are differently affected by H_2O_2, $ONOO^-$ and the combination of H_2O_2 and $ONOO^-$ appear to affect the EF-hands binding sites considerably as a result of shifts in the backbone structure and the spatial positioning of residue side chains. Measuring shifts in distances for O-donor atoms from residues in EF-hands from their native positions shows that there seem to be variations in shifts in distances for residues depending on the effect of H_2O_2, $ONOO^-$ or the combination of both leading in turn to relevant differences. For the **EF-hand 1**, $ONOO^-$ appears to have a more severe effect alone compared to H_2O_2 alone or to the combination of $ONOO^-$ /H_2O_2. After nitration alone co-ordination is lost from 4 out of 5 residues, compared to 3 out 5 for H_2O_2 and $ONOO^-$/H_2O_2 combined **(Figure 54C/D)** .

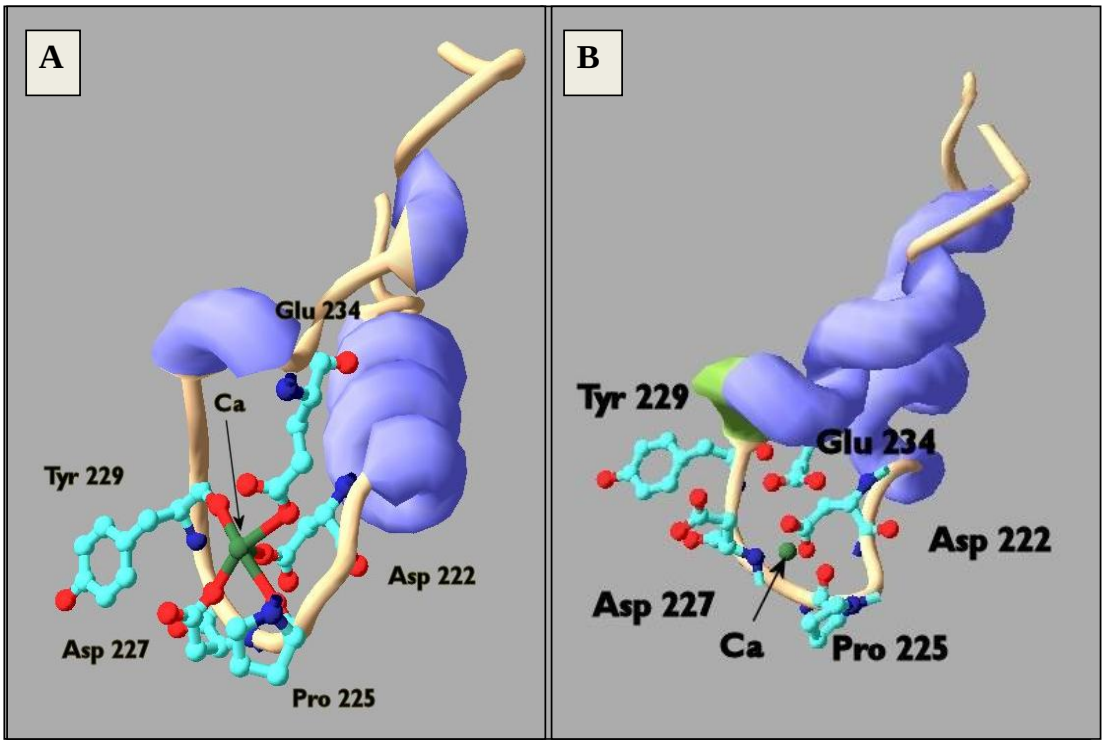

Figure 53: Computer modelling of the1st EF- hands binding site of SPARC in the presence of H_2O_2.

A. 1st EF-hands site in native SPARC. Calcium is bound by 5 O-donor atoms from 5 residues Asp^{222}, Pro^{225}, Asp^{227}, Tyr^{229} and Glu^{234}.

B. The effect of H_2O_2 on the 1st EF-hands binding site. There are substantial shifts in the side chains of calcium co-ordination residues, resulting in loss of co-ordination from over half the O-donor atoms in all cases.

(With kind permission from Dr NCJ Gibbons).

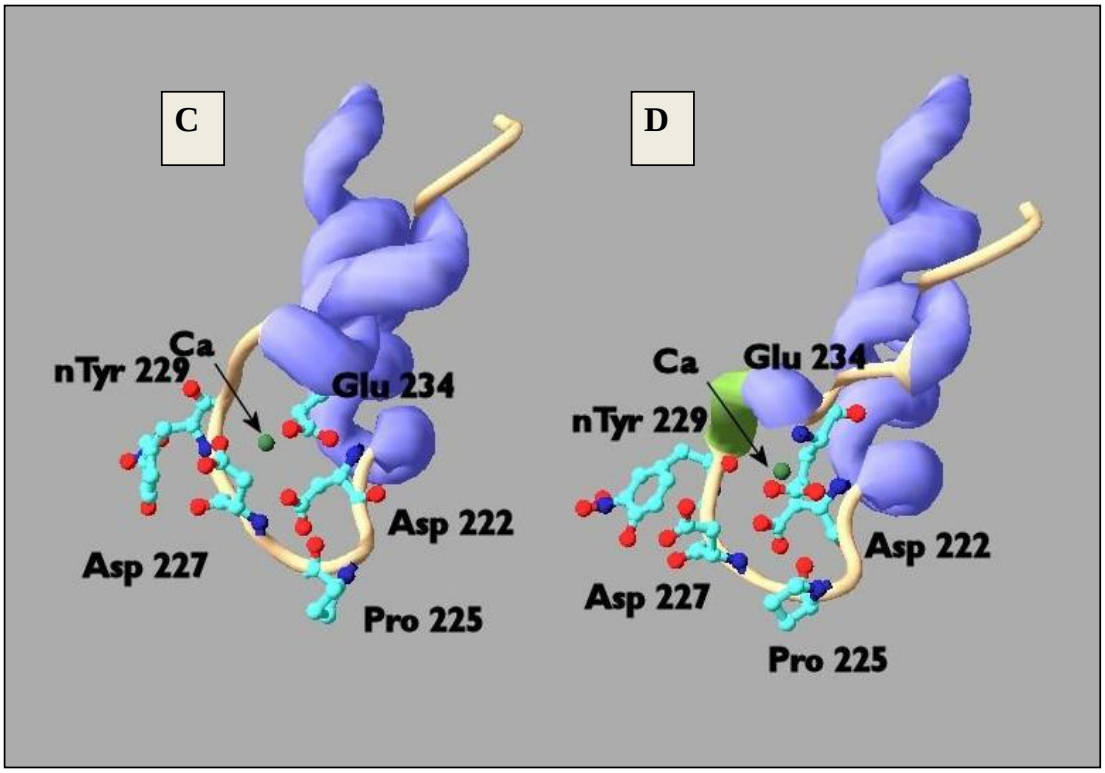

Figure 54: Computer modelling of the 1st EF-hands binding site of SPARC in the presence of $ONOO^-$ and the combination of $ONOO^-$ and H_2O_2.

C. The effect of $ONOO^-$ on the 1st EF-hands binding site of SPARC.

D. The effect of combined $ONOO^-$ and H_2O_2 on the 1st EF-hands binding site of SPARC.

(With kind permission from Dr NCJ Gibbons).

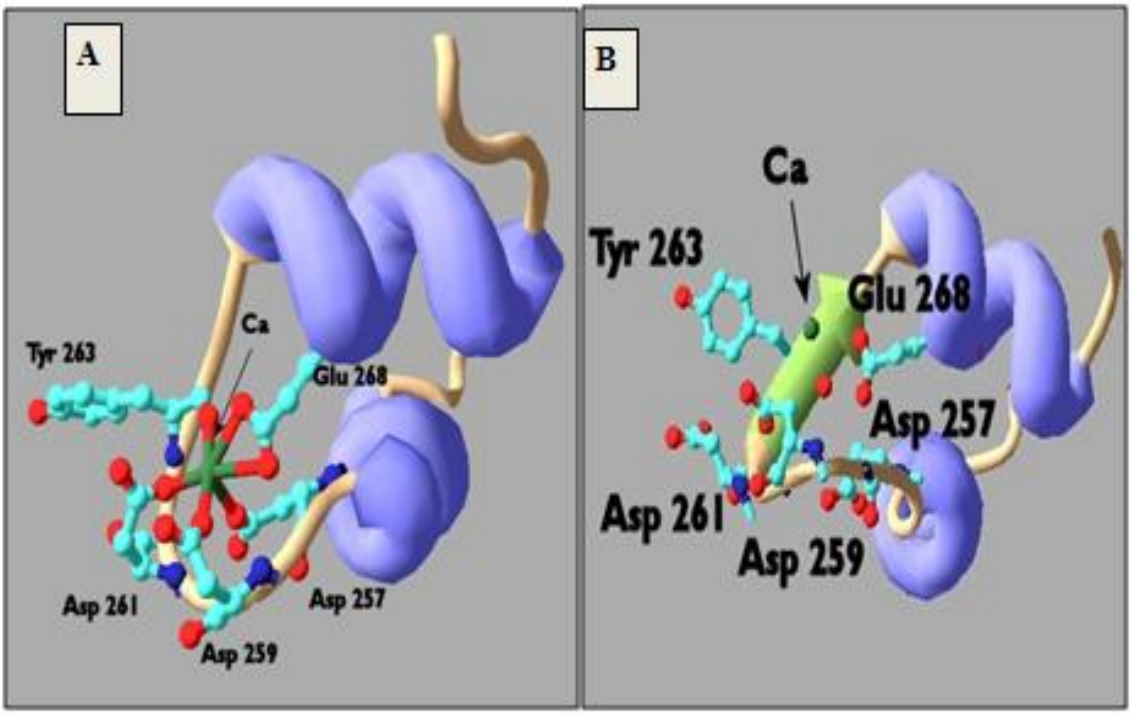

Figure 55: Computer simulation of the 2nd EF- hands binding site of SPARC in the presence and absence of H_2O_2.

A. 2nd EF-hands site in native SPARC. Calcium is bound by 6 O-donor atoms from 5 residues *Asp^{257}, Asp^{259}, Asp261, Tyr^{263} and Glu^{268}*.

B. The effects of H_2O_2 (a) on the 2nd EF-hands binding site of SPARC.

(With kind permission from Dr NCJ Gibbons).

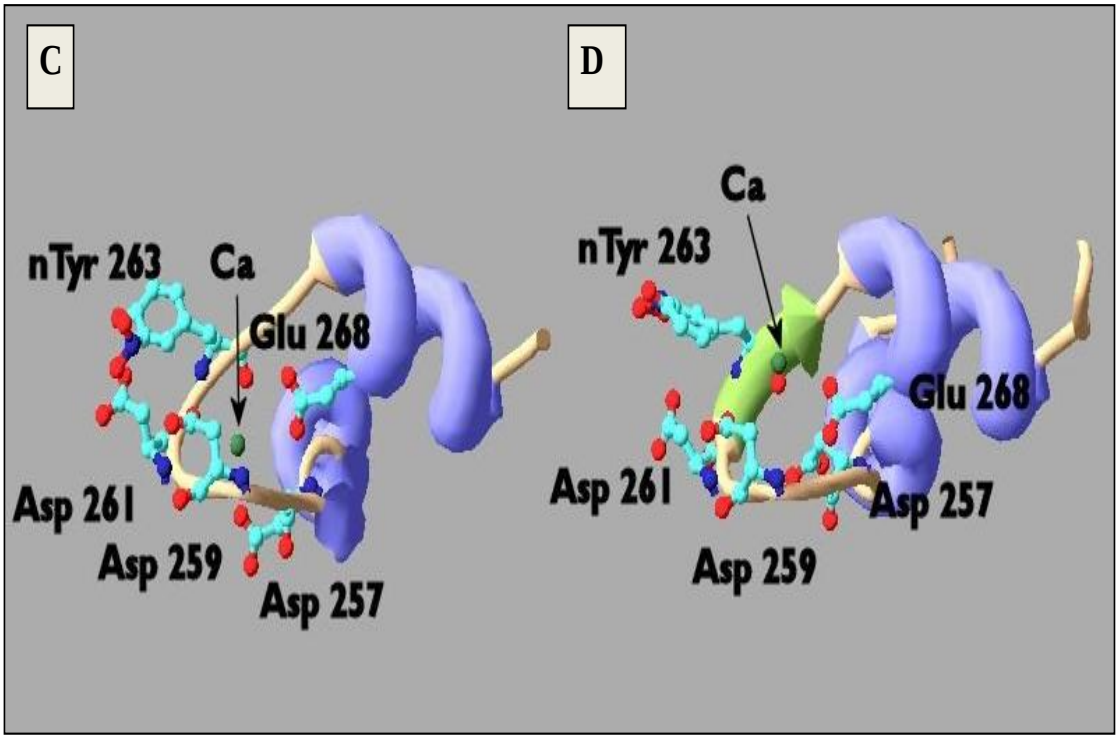

Figure 56: Computer simulation of the 2nd EF-hands binding site of SPARC in the presence of $ONOO^-$ and the combination of $ONOO^-$ and H_2O_2.

C. The effect of $ONOO^-$ on **the 2nd EF-hand** binding site of SPARC

D. The effect of H_2O_2 / $ONOO^-$ combined. While H_2O_2 and H_2O_2/ $ONOO^-$ combined lead to loss of at least 4 out of 6 donor O-atoms, suggsesting the probable loss of calcium, $ONOO^-$ has a slightly weaker effect with the loss of only 3 out of 6 donor atoms. For the **EF-hand 2**, however, the effect of H_2O_2 alone and $ONOO^-/H_2O_2$ combined appear to be more severe than $ONOO^-$ alone with 4 out of 6 O-atoms lost, compared with 3 out of 6 lost for $ONOO^-$**.**

(With kind permission from Dr NCJ Gibbons).

To sum up, the computer modelling results strongly suggest that SPARC is considerably affected by H_2O_2-mediated oxidation and $ONOO^-$ - mediated nitration. Those changes to the structure affect calcium binding and co-ordination via both EF-hands binding sites. As the majority of O-donor atoms in both sites of native SPARC are lost either due to H_2O_2-mediated oxidation and combined oxidation / nitration by H_2O_2/ $ONOO^-$, it is likely that calcium will be lost from both binding sites.

Based on the results, it is tempting to conclude that SPARC can neither induce p53 nor phosphorylate $p90^{MDM2}$ via AKT.

4.3 VEGF-A expression in vitiligo

4.3.1 Significantly up-regulated expression of VEGF-A as SPARC regulator in the epidermal compartment of patients with vitiligo

In an attempt to obtain a more comprehensive view of a possible cellular regulator behind SPARC accumulation in vitiligo, we decided to evaluate VEGF-A levels. Based on previous data it was shown that VEGF-A can induce expression of SPARC in endothelial cells. Furthermore VEGF-A levels showed significant increase in keratinocytes in response to NO and H_2O_2 (Brauchle, et al., 1996; Weninger, et al., 1996; Frank et al., 1999; Kato et al., 2001).

Our own results show strong *in situ* expression of VEGF-A protein throughout the entire epidermis in both lesional (d) and non-lesional (g) skin of patients compared to controls (a) **(Figure 57).** Image analysis of VEGF-A confirms significantly elevated protein expression in vitiligo lesional ($p<0.001$, mean ± SE) and non-lesional ($p<0.001$, mean ± SE) epidermis compared to controls **(Figure 58)**.

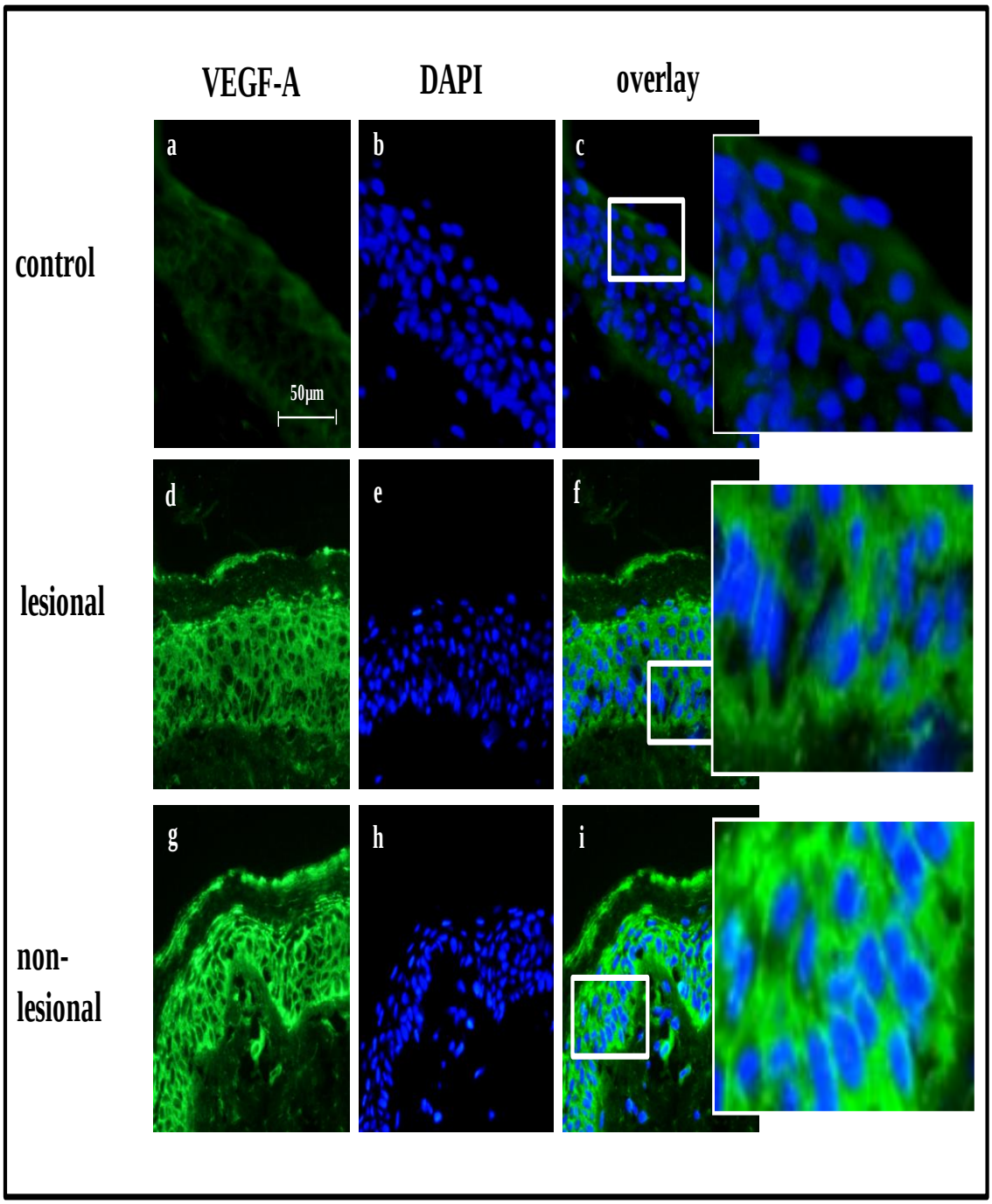

Figure 57: High expression of VEGF-A in the entire epidermal compartment in vitiligo.

Immuno-reactivity (FITC-labelling green) shows increased expression of VEGF in lesional (d) and non-lesional (g) skin of patients compared to healthy controls with skin phototype III (a). Scale bar 50µm. Magnification x 400.

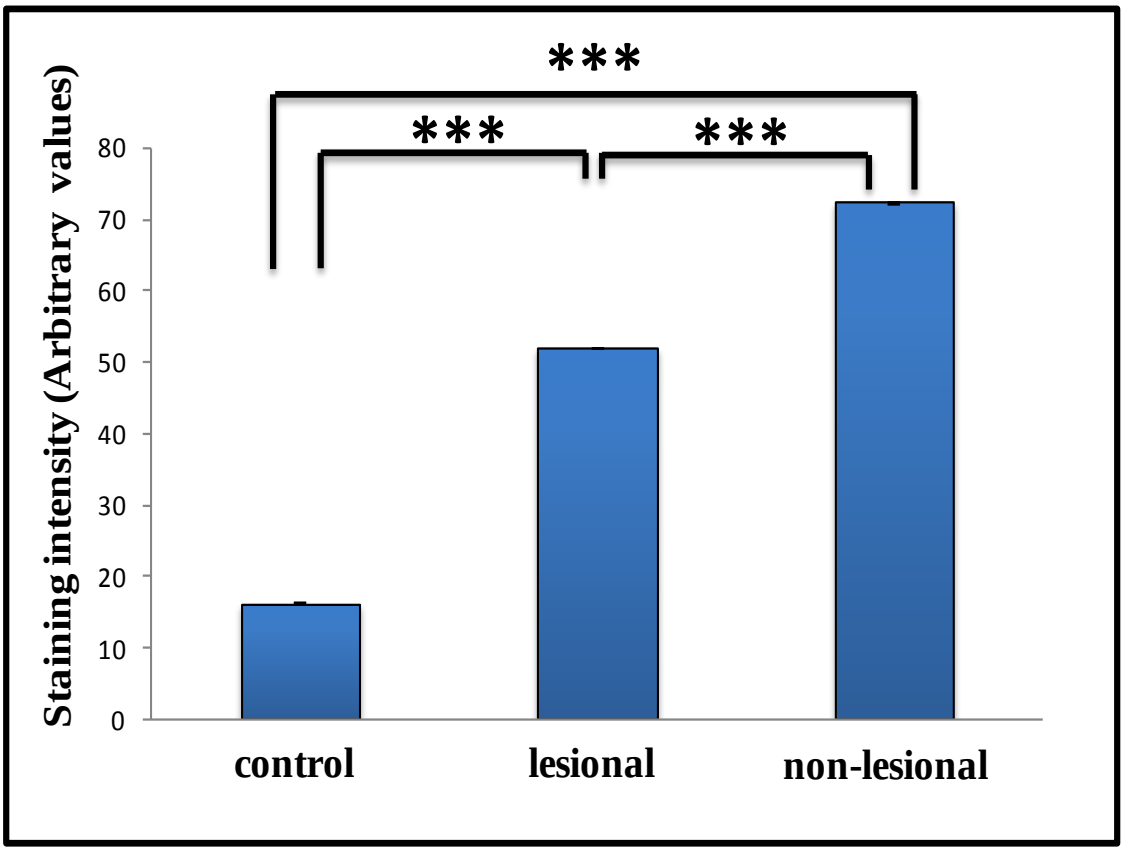

Figure 58: Significantly increased protein expression of VEGF in vitiligo.

Image analysis of the average fluorescence intensity reveals significantly increased levels of VEGF in both vitiligo lesional (n=30: 5 individuals, 6 pictures each) and non-lesional (n=30: 5 individuals, 6 pictures each) skin compared to controls (n=21: 3 individuals, 7 pictures each). (Plots are mean ± SE) (*** $p<0.001$).

4.3.2 More evidence for increased VEGF-A level in vitiligo

In order to further substantiate VEGF-A protein expression, Western blot was carried out. The results confirm up-regulated VEGF-A levels in both lesional (n=3) and non-lesional skin (n=3) of patients compared to healthy controls **(Figure 59 a)**. Image analysis of VEGF-A protein bands in relation to loading control protein GAPDH reveals significantly up-regulated VEGF-A expression in all samples examined **(Figure 59 b, c)**.

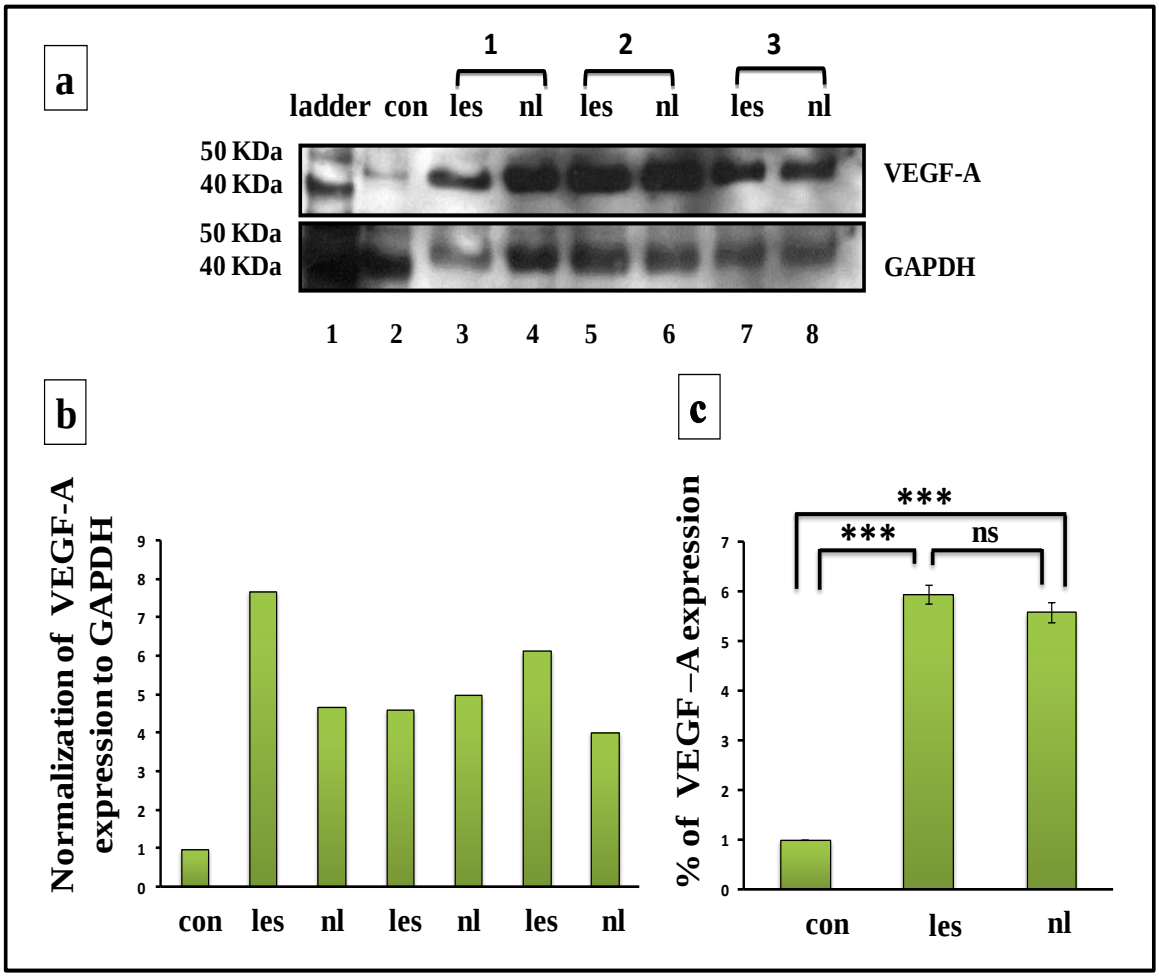

Figure 59: Significantly higher epidermal VEGF-A levels in vitiligo.

(a)Western blot. VEGF-A protein ~43KDa shows increased levels of its expression in both lesional and non lesional skin of patients compared to control. Lane 1 protein ladder, lane 2 control, lanes 3-8 lesional and non-lesional skin tissue extracts from 3 vitiligo patients. GAPDH was used as loading control.

(b) Normalization of VEGF-A expression to the loading control. GAPDH was used to evaluate the expression of the protein in each sample. N.B. levels are increased in all samples examined.

(c) Quantification of the VEGF-A bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals up-regulated expression in both lesional (n=3) and non-lesional (n=3) skin of patients compared to normal healthy control (n=1). (Plots are mean ± SE) (NS $p>0.05$, *** $p<0.001$).

4.3.3 Presence of VEGF-A in epidermal melanocytes

After verification of up-regulated levels of VEGF-A expression in epidermal suction blisters of vitiliginous skin using both *in situ* imuno-fluorescence as well as Western blot, we looked at VEGF-A expression and localization in normal melanocytes and vitiliginous melanocytes under *in situ* conditions using double immune-fluorescence with FITC-labelled VEGF-A and TRITC-labelled NKI / beteb1. Melanocytes of non-lesional skin show stronger VEGF-A expression compared to control skin (**Figure 60)** as indicated by the strong yellow colour in non-lesional skin (**Figure 60 c, i and inserts).**

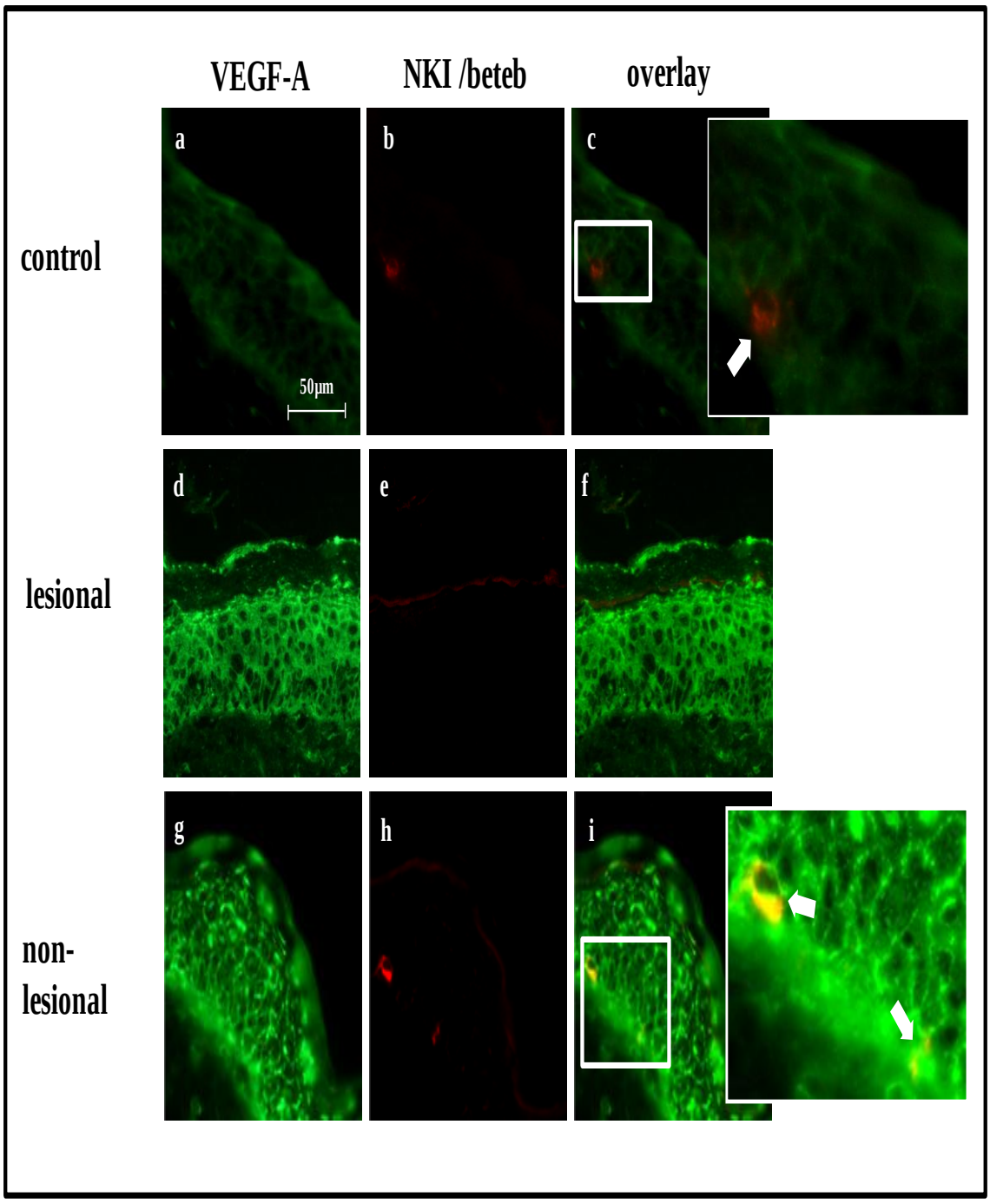

Figure 60: VEGF-A expression in intra-basal epidermal melanocytes of vitiligo skin.

Immuno-reactivity staining (FITC-labelling, green) shows an increase in the expression of VEGF-A in lesional (d) and non-lesional (g) skin of vitiligo compared to control (a) with skin phototype III (a). Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC-labelled VEGF identifies VEGF expression (yellow) in non-lesional skin of vitiligo patients, while it is absent in melanocytes of control skin (c and insert). Magnification x 400. Scale bar 50µm.

We then examined expression in melanocytes under *in vitro* conditions. Unfortunately we had no cells from patients with vitiligo. The results show only background VEGF-A expression in normal melanocytes using double immuno-fluorescence labelling with FITC-labelled VEGF-A and TRITC-labelled NKI / beteb1 **(Figure 61).** To further substantiate this result, we used Western blot. The results confirm absence of VEGF-A expression in these cells **(Figure 62).**

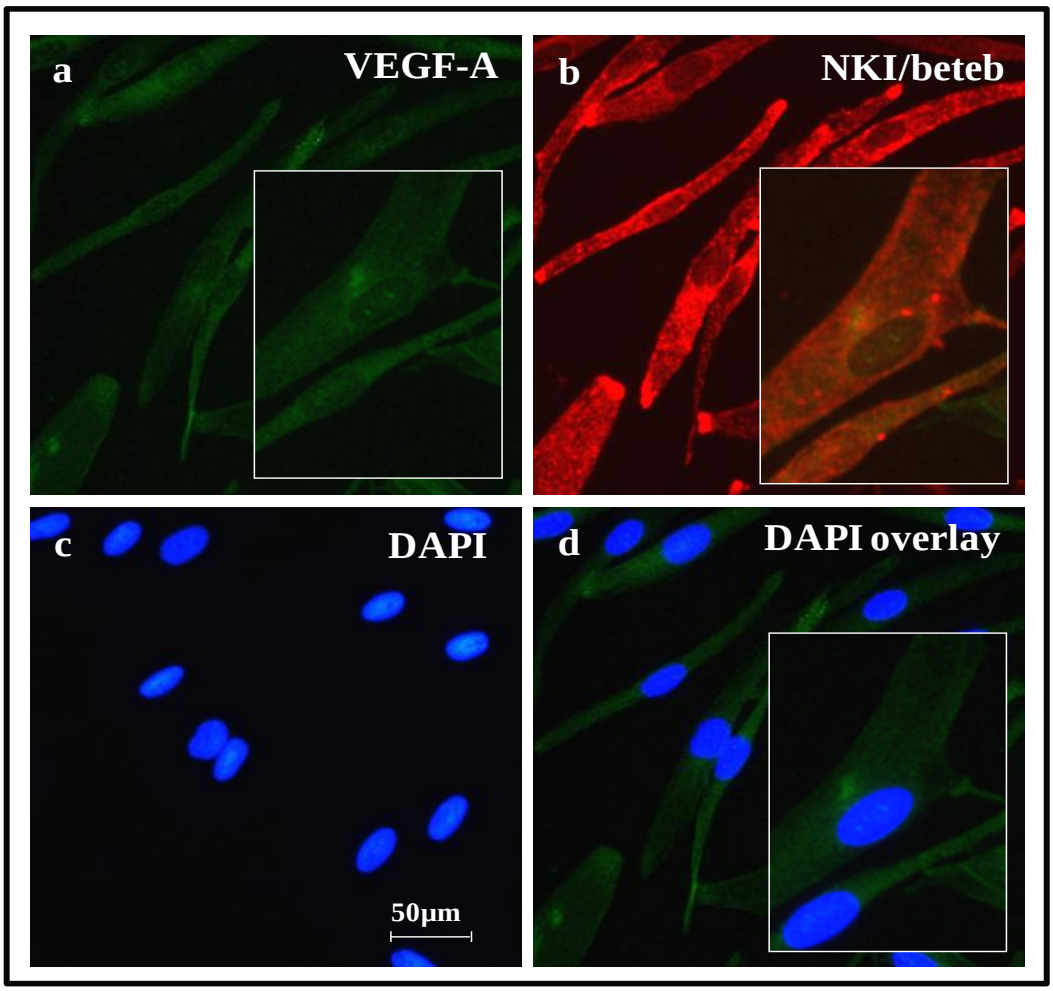

Figure 61: VEGF-A is not expressed in human epidermal melanocytes under *in vitro* conditions.

Immuno-reactivity staining (FITC-labelling, green) shows only background VEGF-A expression in melanocytes. Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC- labelling) and FITC - labelled VEGF-A shows no co-localisation of VEGF-A (b and insert). Magnification x 400. Scale bar 50µm

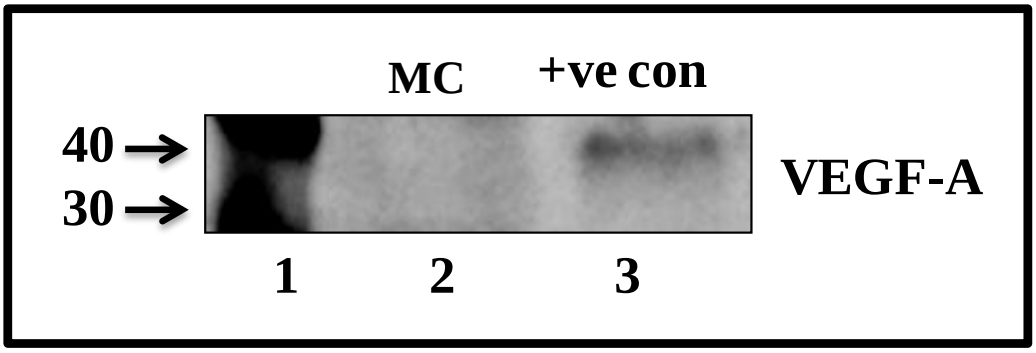

Figure 62: VEGF-A is not detectable in epidermal melanocytes.

Western blot shows no band for VEGF-A in human epidermal melanocytes Lane 1 protein ladder, lane 2 normal human epidermal melanocytes and lane 3 +ve control (melanoma cells).

4.4 TGF-β1 expression in skin of patients with vitiligo

4.4.1 Increased expression of TGF-β1 in the epidermal basal / suprabasal layers of vitiliginous skin

TGF-β1 plays a significant role as a negative regulator for melanogenesis via promoting degradation or inactivation of tyrosinase leading in turn to hypopigmentation. Moreover, it decreases the production of MITF, TRP-1 and TRP-2, affecting melanosome maturation consequently leading to inhibition of melanin formation (Martínez-Esparza et al., 1997; Martínez-Esparza et al., 2001; Kim et al., 2003). In addition it also induces p21 expression through p53-independent mechanisms (Rodeck et al., 1994; Krasagakis et al., 1999; Rodeck et al., 1999; Hoek et al., 2006).

As we showed increased p21 expression in the skin of patients with vitiligo, it was tempting to include a closer look on the expression of TGF-β1 expression in this patient group. Our results show significantly elevated expression of TGF-β1 in the outer cell membrane of the basal and suprabasal layers in lesional (d) skin, while the expression is restricted to the basal layer only in non-lesional (g) skin. There is some expression in the upper layers of vitiligo epidermis. There is only weak basal expression of TGF-β1 in normal control skin (a) (**Figure 63)**. Image analysis of TGF-β1 confirms significantly higher expression in vitiligo lesional and non-lesional epidermis compared to healthy controls (**Figure 64)**.

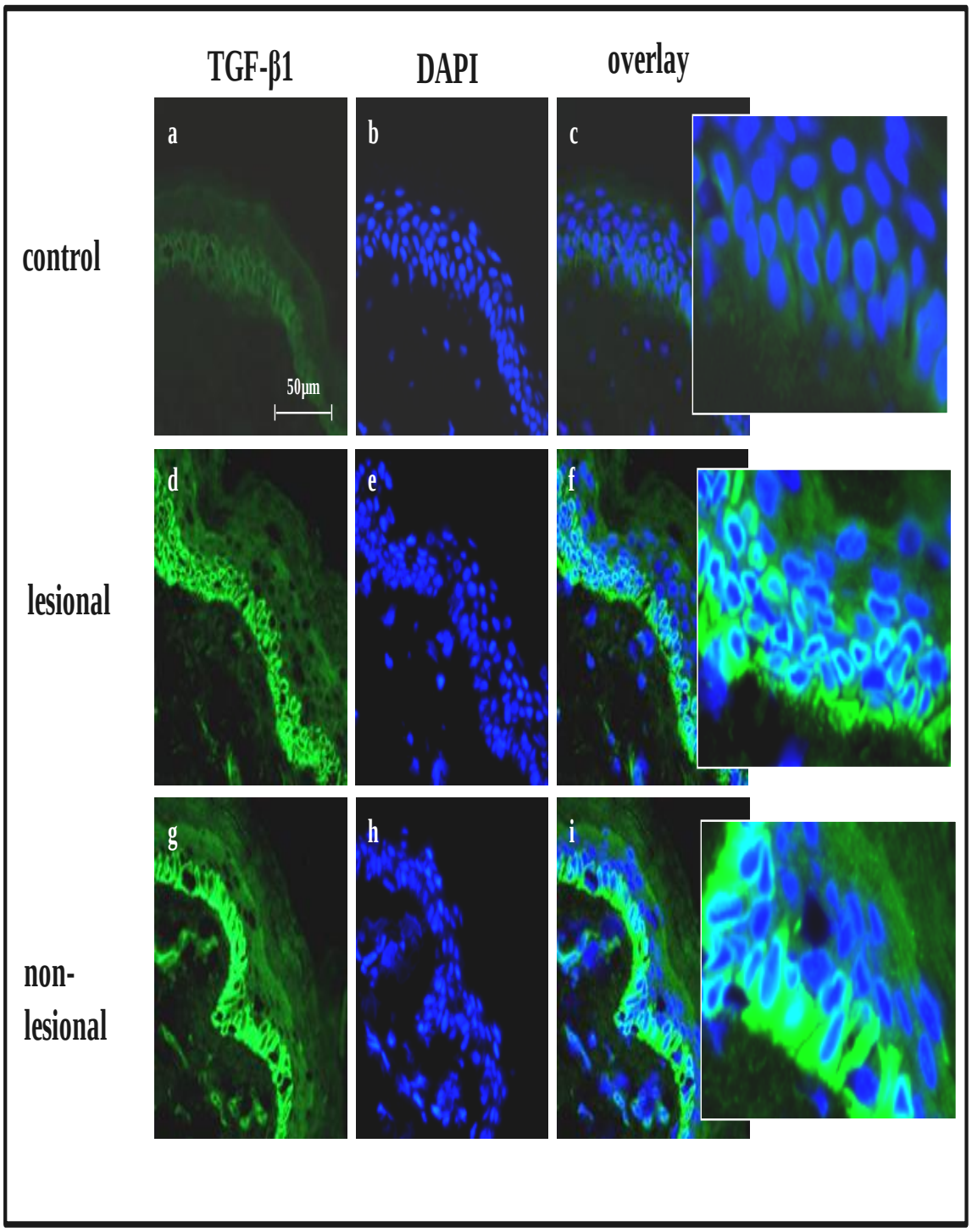

Figure 63: Up-regulated expression of TGF-β1 in the epidermal basal layer of vitiligo patients.

Immuno-reactivity staining (FITC- labelling, green) shows high TGF-β1 expression in the basal suprabasal layers in lesional (d) and non-lesional (g) skin of patients with vitiligo compared to control skin (a). Scale bar 50µm. Magnification x 400.

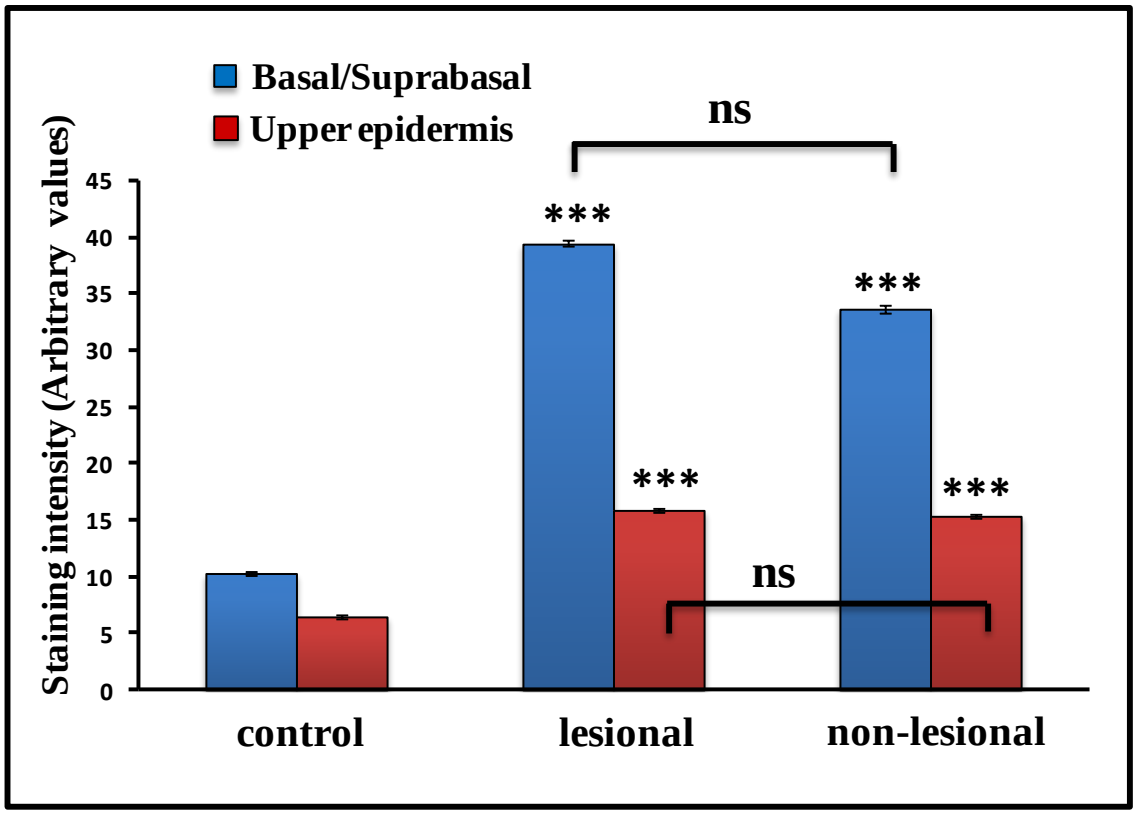

Figure 64: Significantly increased epidermal TGF-β1 expression in vitiligo.

Image analysis of the average fluorescence intensity shows significantly increased levels of TGF-β1 in the basal / suprabasal layers as well as in the upper layers of both lesional (n=40: 5 individuals, 8 pictures each) and non-lesional (n=35: 5 individuals, 7 pictures each) skin, compared to healthy controls (n=21: 3 individuals, 7 pictures each). (Plots are mean ± SE) (*** $p<0.001$)

4.4.2 TGF-β1 in epidermal melanocytes

After our observation of high *in situ* TGF-β1 protein expression throughout the entire epidermis of patients with vitiligo, the presence and localization of this protein was investigated in normal and vitiliginous melanocytes under *in situ* conditions. Interestingly, in skin of patient with vitiligo, melanocytes from non-lesional skin showed some TGF-β1, while it was absent in melanocytes of control skin (**Figure 65). N.B.** It was also absent in a detached melanocyte in lesional skin.

Normal melanocytes under *in vitro* conditions showed only background TGF-β1 expression. Some expression is present in nucleoli **(Figure 66).** Western blot fosters absence of this protein in normal human epidermal melanocytes utilised **(Figure 67).**

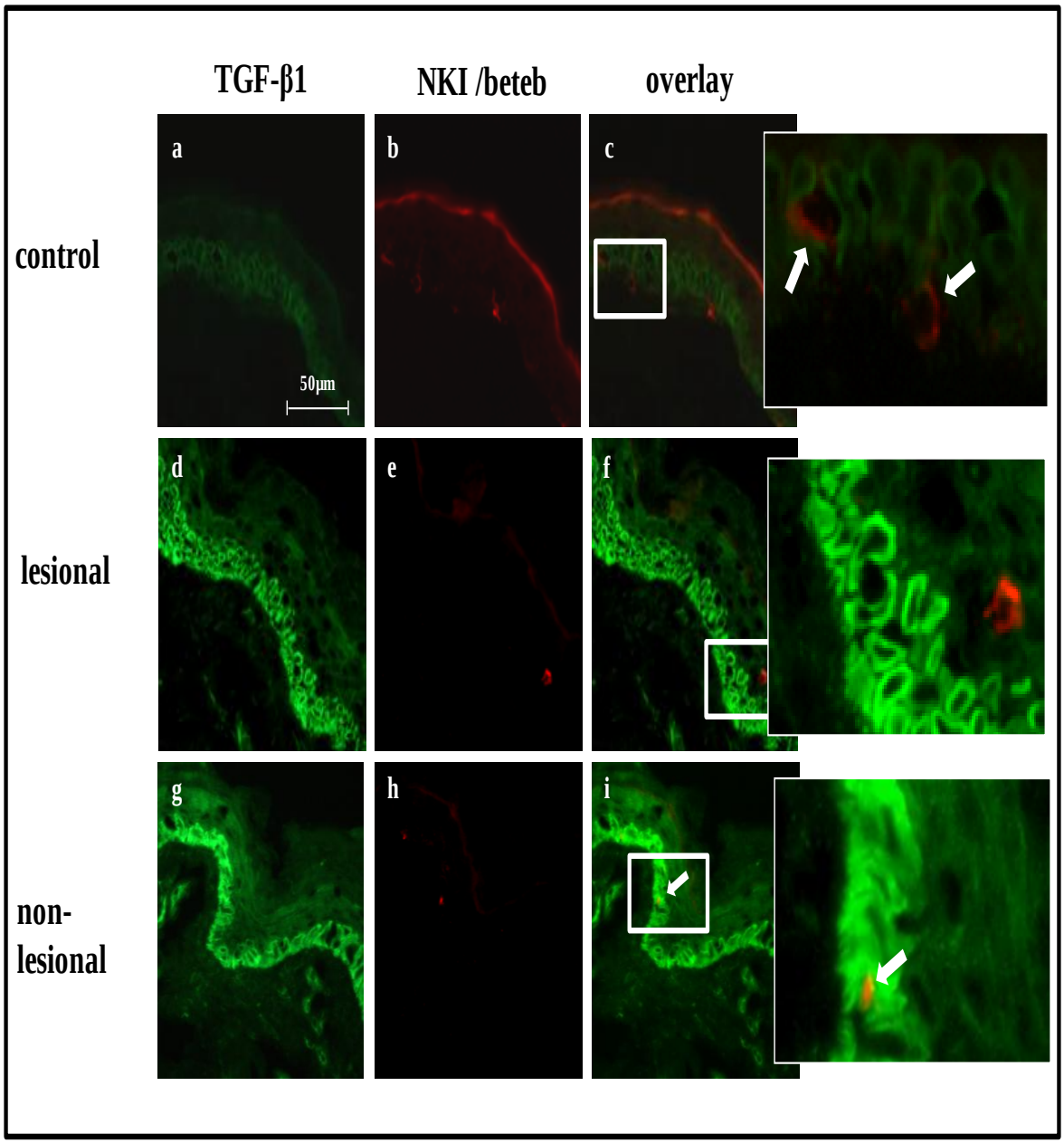

Figure 65: TGF-β1 expression in basal and suprabasal layers of patients with vitiligo.

Immuno-reactivity staining (FITC-labelling, green) shows high expression of TGF-β1 in lesional (basal and suprabasal layers) (d) and non-lesional (g) skin of patients with vitiligo compared to control (a) NKI / beteb1 positive TRITC-labelled melanocytes of non-lesional skin (b; e; h) show some expression of TGF-β1 (in yellowish orange; i and insert) compared to melanocytes of lesional and control skin. **NB**. One detached suprabasal NKI / beteb 1 positive/ TGF-β1 negative melanocyte in lesional vitiligo (f and insert). Scale bar 50µm. Magnification x 400.

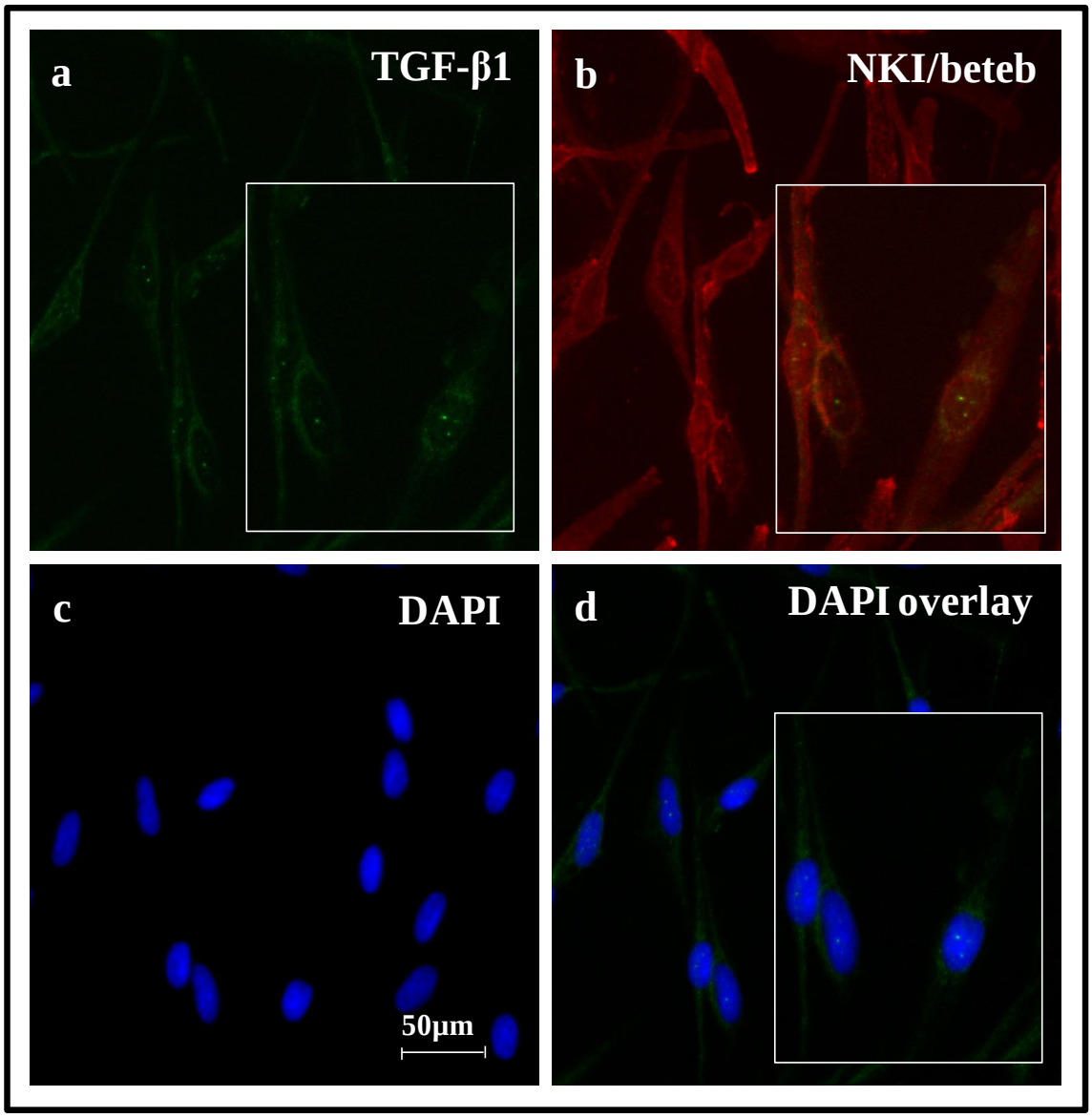

Figure 66: *In vitro* expression of TGF-β1 in human epidermal melanocytes.

Immuno-reactivity staining (FITC-labelling, green) shows little TGF-β1 expression in melanocytes. Overlay of TGF-β1 positive epidermal melanocytes (TRITC-labelling, red) and FITC - labelled TGF-β1 shows no co-localisation of TGF-β1 (b and insert). Magnification x 400. Scale bar 50µm.

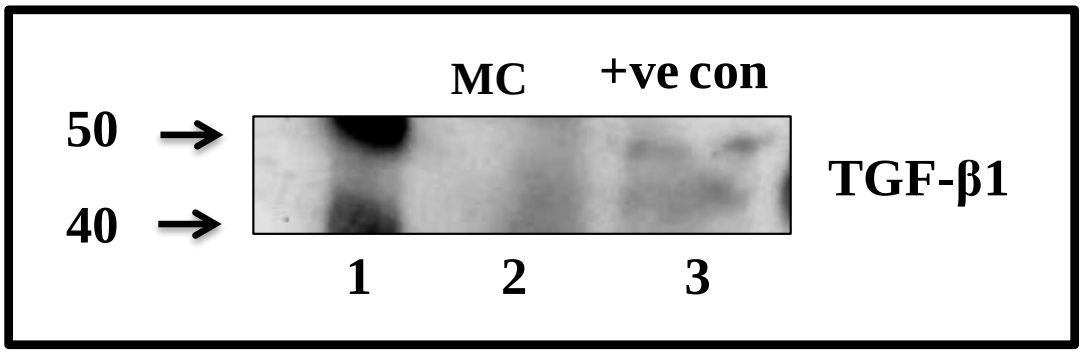

Figure 67: TGF-β1 is absent in epidermal melanocytes.

Western blot confirms absent expression of TGF-β1at ~44KDa in human epidermal melanocytes. Lane 1 protein ladder, lane2 normal human epidermal melanocytes and lane 3 is +ve control (melanoma cells).

4.4.3 Epidermal TGF-β1 is nitrated in vitiligo

Double immuno-fluorescence staining of FITC-labelled TGF-β1 and TRITC-labelled 5-nitro-tyrosine reveals significant co-localisation between the highly expressed TGF-β1 protein and 5-nitro-tyrosine in lesional and non-lesional skin of patients. This elevated expression of nitrated TGF-β1 is pronounced in the basal and suprabasal layer of lesional and non-lesional skin in patients with vitiligo **(Figure 68)**.

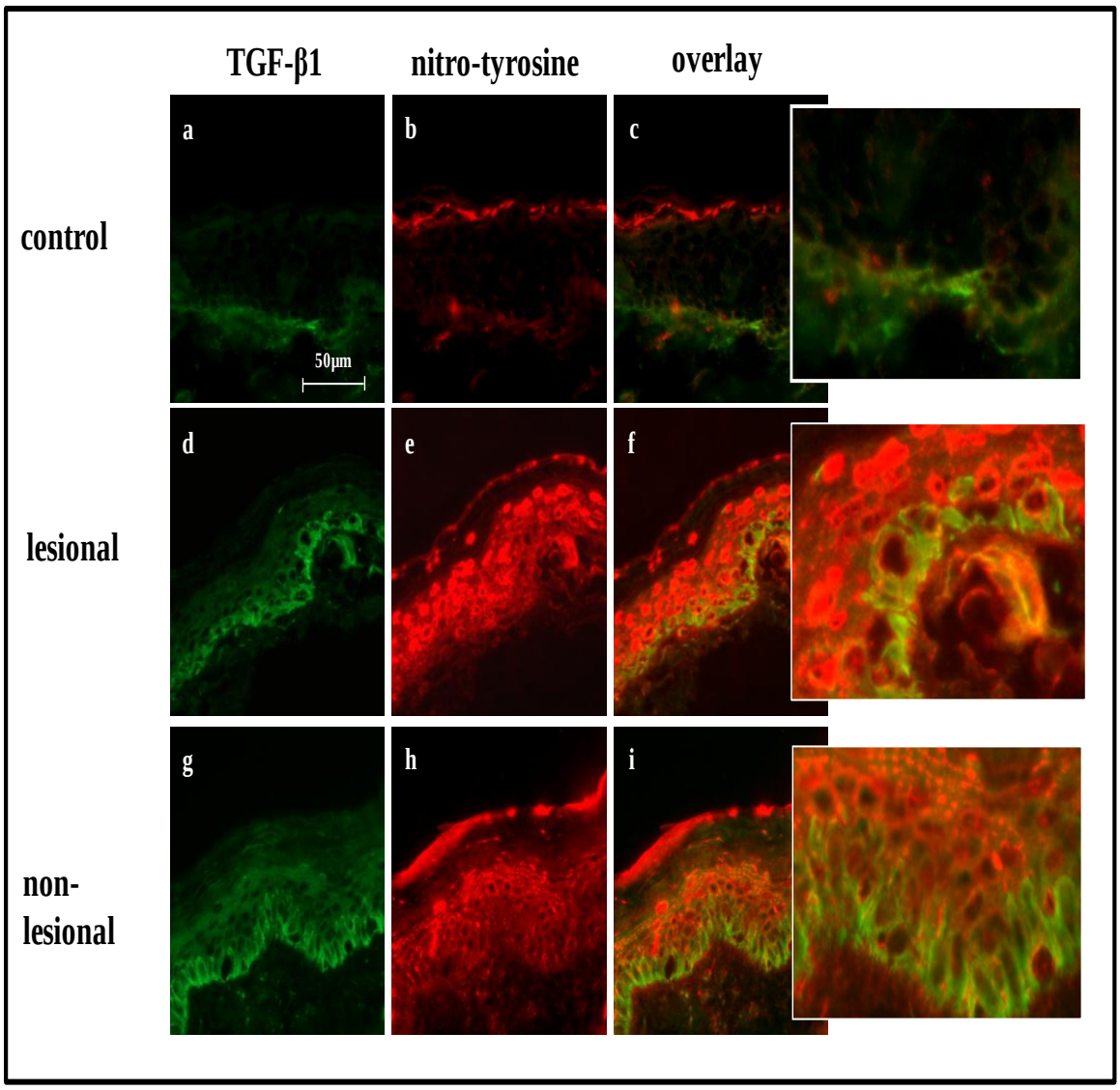

Figure 68: Up--regulated *in situ* expression of nitrated TGF-β1 in the basal layer of vitiligo.

Immuno-fluorescence reactivity of TRITC-labelled 5-nitro-tyrosine and FITC-labelled TGF-β1 represents nitrated TGF-β1 in vitiligo lesional (f) and non-lesional (i) skin. Overlay of TGF-β1 and nitrated tyrosine identifies the presence of nitrated TGF-β1 through the epidermis of vitiligo lesional and non-lesional skin (f, i respectively) compared to healthy controls (c) (skin phototype III, Fitzpatrick classification). Scale bar 50µm. Magnification x 400.

5. Discussion

To date there is convincing evidence that H_2O_2-mediated oxidative and $ONOO^-$ - mediated nitrative stress in the entire epidermis of patients with vitiligo is implicated in the pathogenesis of this disease (for review see Schallreuter, 2014). In this context oxidation / nitration of proteins and peptide structures at various amino acid residues, including L-methionine, L-tryptophan, L-cysteine, seleno cysteine and L-tyrosine has been widely documented, leading in turn to complete or partial loss of function (Rokos et al., 2002; Gillbro et al., 2004; Hasse et al., 2005; Schallreuter et al., 2005b; Spencer et al., 2005; Chavan et al., 2006; Elwary et al., 2006; Gibbons et al., 2006; Schallreuter et al., 2008a; Shalbaf et al., 2008; Wood et al., 2009; Salem et al., 2009; Schallreuter et al., 2012 a; b ; Schallreuter, 2014)

Moreover, 10^{-3}M concentrations of H_2O_2 can trigger cellular lipid peroxidation, leading in turn to appearance of cellular vacuolation in this disease (Moellmann et al., 1982; Bhawan and Bhutani, 1983; Boissy et al., 1991; Nordllund and Ortonne, 1992; Schallreuter., 1999a; Schallreuter et al., 1999b; Tobin et al., 2000).

H_2O_2-mediated oxidation affects epidermal antioxidant enzymes disturbing in turn the entire antioxidant defence machinery for prompt removal of oxidative toxic intermediates (Aronoff, 1965; Schallreuter et al., 1991b; Schallreuter 1999a; Schallreuter et al., 1999b; Schallreuter, 2005a; Maresca et al., 2006; Wood and Schallreuter, 2006; Schallreuter et al., 2007d; Schallreuter and Elwary, 2007a; Spencer et al., 2007; Shalbaf et al., 2008; Wood et al., 2008; Salem et al., 2009, Schallreuter et al., 2012 a; b, Schallreuter, 2014).

The enzyme catalase, amongst others, is one important member of the antioxidant system. Under normal conditions catalase promotes degradation of H_2O_2 in to H_2O and O_2 (Aronoff, 1965; Schallreuter et al., 1991b).

Low catalase levels have been documented in the entire epidermis in vitiligo (Schallreuter et al., 1991b). In this context it has been shown that H_2O_2 affects the porphyrin ring of the enzyme in addition to oxidation of methionine and tryptophan residues in the active site and the cofactor NADPH-binding site, leading in turn to enzyme degradation and loss of functionality.Here it is of note that catalase expression in vitiligo emerged as an excellent biomarker for oxidative stress (Aronoff, 1965; Gibbons, et al., 2006; Maresca et al., 2006; Wood and Schallreuter, 2006; Salem et al., 2009; Schallreuter et al., 2012a; b; Schallreuter 2014). H_2O_2 can also deactivate thioredoxin reductase (TR), another important enzyme for turnover of 1. H_2O_2 to H_2O and O_2 and 2. for turnover of nitric oxide (NO) to hydroxyl amine. One consequence is accumulation of NO which reacts with $O_2^{\cdot}$, forming peroxinitrite ($ONOO^-$). This radical nitrates tyrosine residues to 5-nitro-tyrosine in different protein structures, contributing in turn to more imbalances in functionality (van der Vliet et al., 1994; Oury et al., 1995; Groves, 1999; Salem et al., 2009).

In this context it is of interest that both, H_2O_2 and NO, increase the expression of VEGF-A in keratinocytes (Brauchle, et al., 1996; Frank et al., 1999). Moreover, this growth factor enhances SPARC expression and activity in human vascular endothelial cells (Weninger et al., 1996; Kato et al., 2000). Expression and activation of SPARC has been reported in melanoma to down-regulate as well as inactivate p53, leading in turn to inhibition of p21 mediated cell cycle arrest (Fenouille et al., 2011a;b). Given our documented up-reguated wild type p53 levels in classical vitiligo together with up-regulated p76 $^{MDM-2}$ (Perry et al., 2000; Schallreuter et al., 2003; Salem et al., 2009), it was tempting to investigate levels and activity of other important p53 regulators .

Our study included the p53-negative regulator, MDM4 and its phosphorylated form, MDM4phospho. Moreover, we looked at expression and signalling of SPARC and its possible contribution in stabilizing wild type p53 in the skin of patients with vitiligo.

Here it becomes of interest that SPARC induces TGF-β (Bassuk et al., 2000). As TGF-β acts as negative regulator of melanogenesis via decreasing maturation of melanosomes through degradation or inactivation of tyrosinase in addition to inhibition of MITF, TRP-1 and TRP-2 production (Martínez-Esparza et al., 1997; Martínez-Esparza et al., 2001; Kim et al., 2003), we hypothesized a possible link between SPARC and the depigmentation process. One rationale behind this idea was, that patients with vitiligo lack protection against UVR threats due to absence of melanin in addition to massive epidermal oxidative stress. In this context up-regulated p53 was proposed as one possible mechanism behind a normal risk for non-melanoma skin cancer and lack of extensive skin photo-damage (Calanchini-Postizzi and Frenk, 1987; Schallreuter et al., 2002; Salem et al., 2009).

Taken together, to get a better understanding on the permanent up-regulated wild type p53 in vitiligo, we followed epidermal catalase, p53, p21, p76 ^{MDM2}, MDM4, MDM4phospho and SPARC expression in the same patient's skin. Moreover, as VEGF-A induces SPARC (Kato et al., 2000; Weninger, et al., 1996) and VEGF-A expression is enhanced in human epidermal keratinocytes by NO and H_2O_2, (Brauchle, et al., 1996; Frank et al., 1999), we included this signal and its possible role as a regulator for increased SPARC and p53 levels in our study. Our first approach involved *in situ* investigation of the above protein panel in comparison to healthy controls. In addition Western blot analysis was utilized for assessment of protein levels. Moreover, our analyses included *in situ* and *in vitro* studies of epidermal melanocytes and *in vivo* FT-Raman spectroscopy as well as computer simulation.

Low epidermal catalase levels and high 5-nitro-tyrosine expression support the oxidative stress theory in vitiligo

Our *in situ* studies of epidermal catalase expression in patients and controls confirm significantly down-regulated expression of the enzyme throughout the entire epidermal compartment, in lesional and non-lesional skin **(Figure 14, 16)**. Together with our results from *in vivo* FT-Raman spectroscopy, which confirmed the presence of H_2O_2 in 10^{-3}M concentration, identified by the O=O stretch at 875cm^{-1} **(Figure 19)**, low epidermal catalase expression levels reflect H_2O_2–mediated oxidation of the protein in these patients (Schallreuter et al 1991b, Schallreuter et al 1999b; Schallreuter et al., 2008a, Schallreuter, 2014). Here it is of note, that catalase levels follow melanogenesis in normal melanocytes (Maresca et al., 2007). Our data, presented in this thesis, confirm high *in vitro* and *in situ* catalase expression in melanocytes of healthy control skin **(Figure 18),** while expression in melanocytes of non-lesional skin of patients with vitiligo is almost absent **(Figure 17)**.

Moreover, the presence of these massive epidermal H_2O_2 concentrations in our vitiligo samples is accompanied with significantly higher 5-nitro-tyrosine levels **(Figure 50).** Hence, our results are in agreement with previous data, published by Salem and colleagues, who were the first to document significant increased $ONOO^-$ levels in both lesional and non-lesional skin of patients with vitiligo (Salem et al., 2009).

To sum up, characterisation of our vitiligo samples confirmed the presence of massive H_2O_2 and $ONOO^-$ accumulation in the entire epidermal compartment of vitiligo in all patients investigated.

Confirmation of up-regulated epidermal functioning wild type p53 together with up-regulated p21 expression throughout the entire epidermis in vitiligo

Given, the dramatic epidermal H_2O_2 / $ONOO^-$ accumulation together with significantly lower / almost absent catalase levels in vitiligo lesional and non-lesional skin (Aronoff, 1965; Schallreuter et al., 1991b; Schallreuter et al., 1999b; Schallreuter, 2004b; Wood and Schallreuter, 2006; Schallreuter and Elwary, 2007a; Schallreuter et al., 2008a; Spencer et al., 2007; Shalbaf et al., 2008; Wood et al., 2008; Salem et al., 2009; Schallreuter et al., 2012 a;b; Schallreuter, 2014), we re-examined *in situ* expression of both, p53 and p21, in the epidermal compartment of these patients and compared those to healthy controls. Our results confirm high expression of cytoplasmatic and nuclear p53 throughout the entire epidermis **(Figures 20, 22).** This expression is also pronounced in melanocytes of non-lesional skin compared to melanocytes in healthy controls **(Figure 23).** Here it is of note that p53 levels are not affected by H_2O_2-reduction after treating patients with a topical application of narrowband UVB-activated pseudocatalase PC-KUS (Schallreuter et al., 2003; Salem et al., 2009). This observation suggests that H_2O_2-levels in the epidermis of these patients, although reduced, could still be high enough to trigger p53 transcription (Vile, 1997). Moreover, combination of both, $ONOO^-$ and H_2O_2, increases the efficiency of DNA-binding capacity of p53 in vitiligo, which is not the case, if only $ONOO^-$ is present (Salem et al., 2009).

According to previous reports, localisation of p53 in the nuclei of epidermal cells may refer to its stabilization. Increased p53 stability after continous oxidative stress prevents binding of the p53 negative regulator MDM2 to p53 after its phosphorylation at or near

the MDM2-binding domain, which would prevent p53 ubiquination, leading in turn to accumulation of p53 in the nuclei (Giaccia and Kastan, 1998; Chehab et al., 1999).

Therefore, our question of interest was, whether p53-functionality as a transcriptional factor via binding to its target genes was affected in vitiligo. For this purpose we studied the expression of p21 (cyclin-dependent kinase inhibitor 1), a protein member of the cell cycle arrest orchestra. It is well established that p21 interferes with the cell cycle at the G1-phase (Kuerbitz et al., 1992; el-Deiry et al., 1993) and G2 - phase (Lin and Lowe, 2001) providing in turn time for DNA-repair (Hartwell, 1992; Hartwell and Kastan, 1994).

Our *in situ* immuno-fluorescence labelling of p21 as well as Western blot data confirm up-regulated p21 expression and levels throughout the entire epidermis in vitiligo **(Figures 24, 26).** Epidermal melanocytes of non-lesional skin show significantly higher expression of p21 **(Figure 24).** These findings are in agreement with the previously published data by Salem and colleagues, who showed increased p21 levels in vitiligo skin (Salem et al., 2009). Clearly, this result points to the positive switch for p53-downstream signaling and the efficiency of p53 in enhancing cell cycle arrest at G1 and G2 phases, preventing in turn proliferation of injured cells and giving time for DNA-repair via up-regulated base excision repair (BER) as reported earlier (Salem et al., 2009).

Taken together, our data confirm once more up-regulated functioning p53 in vitiligo.

In vitiligo epidermal cells induce p76^{MDM2} expression, hindering in turn p53 degradation

As mentioned above, p90^{MDM2} controls p53 stability and activity via two distinct mechanisms. On one hand, the protein causes a hold in the p53-dependent

transcriptional machinery via binding to the N-terminal domain of p53, preventing in turn binding of gene expression inducing factors. On the other hand, $p90^{MDM}$ controls ubiquitination of p53 and targets the protein for proteasomal degradation (Oliner et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997; Honda and Yasuda, 2000). The family member $p76^{MDM2}$ lacks the p53 binding domain of $p90^{MDM2}$ (Chen et al., 1993; Olson et al., 1993; Haines et al., 1994; Saucedo et al., 1999). By contrast to other MDM2 family members, $p76^{MDM2}$ acts positively towards p53. It antagonizes p53-regulation by $p90^{MDM2}$ via inhibition of $p90^{MDM2}$ binding to p53, without affecting $p90^{MDM2}$levels (Perry et al., 2000; Giglio et al., 2010). Hence, $p76^{MDM2}$ prevents p53 degradation. However, it is of note, that $p76^{MDM2}$ binds to p53 mRNA, promoting in turn efficient translation of mRNA (Naski et al., 2009). These data motivated us to re-investigate the expression of $p76^{MDM2}$ in our characterised patient group. Our *in situ* immuno-fluorescence results, together with the Western blot data **(Figure 28, 30)**, proved significantly higher $p76^{MDM2}$ protein levels in the entire epidermis of patients which are absent in healthy skin. Hence, our data support the overriding control via $p76^{MDM2}$ in maintaining stability and activity of p53 through prevention of $p90^{MDM2}$ - p53 binding.

Taken together, our data are in agreement with previously published results by Salem and colleagues, who documented for the first time significantly up-regulated $p76^{MDM2}$ expression in lesional and non-lesional vitiligo skin, accompanied by high levels of p53 and p21 (Schallreuter et al., 2003; Salem et al., 2009). Once more, it is tempting to invoke these data as an explanation for the low incidence of solar-induced non-melanoma skin cancer as well as absence of increased photo-damage, photo-aging, and low sunburn sensitivity in these patients, despite the presence of massive oxidative stress in the skin together with the absence of protecting pigment (Calanchini-Postizzi and Frenk, 1987; Schallreuter et al., 2002, Salem et al., 2009, Teulings et al 2013).

Up-regulated MDM4 / MDM4phospho levels support up-regulated functioning p53 expression in vitiligo – a novel result

After confirmation of stability and functionality of p53 in association with high $p76^{MDM2}$ and normal $p90^{MDM2}$ levels, we then looked in to MDM4 interaction as MDM4 controls p53 levels through inhibition of $p90^{MDM2}$-mediated p53 degradation. Moreover, MDM4 directly affects p53 activity via disturbing its transcriptional machinery (Honda et al., 1997; Sharp et al., 1999; Fang et al., 2000; Jackson and Berberich, 2000; Stad et al., 2000; Pan and Chen., 2003; Danovi et al., 2004).

Our *in situ* immuno-fluorescence results demonstrate for the first time significantly increased MDM4 expression throughout the entire epidermis in both lesional and non-lesional skin **(Figure 31)**.This result was confirmed by Western blot in lesional skin of three different patients **(Figure 33)**. These data, together with the results of increased p21 expression **(Figure 26)** in the skin of the same three patients in both lesional and non-lesional skin, could point to MDM4-independent control of p53 transcriptional activity. On one hand, our data are in sharp disagreement with reports by Danovi and colleagues, who demonstrated a major role of over-expressed MDM4 for p53 tumour suppressor activity inhibition in MDM4-infected cultures by showing a striking reduction in p21 expression (Danovi et al., 2004). On the other hand, our results are in agreement and supported by data published in the same year by Mancini and colleagues. These authors demonstrated under stress conditions in non-tumour cells, that MDM4 over-expression enhances p53 protein levels and transcriptional activity together with increased dissociation of p53 from its negative regulator $p90^{MDM2}$ (Mancini et al., 2004).

This scenario portrays exactly the condition in vitiligo with

- the presence of oxidative stress suffering cells
- up-regulated wild type p53
- normal $p90^{MDM2}$ levels
- efficient p53-transcriptional machinery,
- supported by high p21 levels
- significantly up-regulation of MDM4

In this context it was suggested that under stress conditions endogenous MDM4 is stabilizing stress-induced p53 due to its antagonism towards MDM2 (Barboza et al., 2008). Based on these data, it is tempting to speculate that different MDM4 actions may exist between tumour and non-tumour- stressed cells.

Moreover, it has been shown that UV-radiation affects MDM4 activity via phosphorylation (Jin et al., 2006). As UV induces *de novo* melanogenesis, but patients with vitiligo hold a partial loss of their inherited skin colour and *de novo* UV- regulated epidermal pigment, we decided to investigate phosphorylation of MDM4 for further elucidation of p53 accumulation in our characterised vitiligo samples. As shown by our Western blot results, we found variable phosphorylation levels in MDM4 protein amongst the three patients analysed **(Figure 39).** Only one of them showed elevated MDM4phospho in lesional and non- lesional skin, while expression levels are very low in the two other patients. Here it is important to note, that the protein levels were originating from the same Western blots after stripping. Hence, the variable results are real with no artefacts. Consequently we analysed the MDM4 / MDM4phospho ratio in these samples, hoping for a further answer. Analyses of the individual results (**Table 5)** reveal higher MDM4 levels than the corresponding phosphorylated protein in patients compared to healthy controls, suggesting, that not all MDM4 is phosphorylated in each

patient. However, the MDM4 / MDM4phospho ratio is higher in all patients examined. Given, we accept the results of Mancini's and Barboza's group, who reported and confirmed that MDM4 **in stressed cells** positively regulate p53, then our data imply that some epidermal MDM4 protein is still un-phosphorylated. As a consequence MDM4 could still function to antagonise $p90^{MDM2}$ binding to p53, providing in turn p53 - stabilisation and activity. Clearly in vitiligo oxidative stressed cells are present in the epidermal compartment. On the other hand if MDM4 would function as a negative p53-regulator in a tissue or cell type-independent manner, the remaining un-phosphorylated MDM4 could be inhibited / lost its control on p53. In this context it was reported that hetero-dimerization of $p90^{MDM2}$ and MDM4 is crucial for degradation of p53 (Linke et al., 2008). Whereas Giglio and colleagues in 2010 suggested that $p76^{MDM2}$ interferes with MDM complex formation, reducing in turn its degradation activity on p53 (Giglio et al, 2010).

Our results revealed, almost absent, expression of both MDM4 and MDM4phospho in melanocytes *in vitro* and *in situ* in healthy controls **(Figures 34, 35, 36, 40, 41, 42).** These results are in agreement with recent data, where very low and / or undetectable MDM4 expression in these cells was reported (Gembarska et al., 2012). Surprisingly, expression of these proteins is high in melanocytes of non-lesional skin in our patients, **(Figures 34, 40)**, associated with increased p53 **(Figure 23)** and p21 expression **(Figure 27)**. Taken together, MDM4 function in vitiligo may proceed through one of the following mechanisms.

Inhibition of negative regulatory function towards p53 via

- impaired heterodimer formation with $p90^{MDM2}$
- preventing p53 degradation based on high levels of $p76^{MDM2}$, hindering in turn MDM complex formation and MDM4 binding to p53.

The overall result is a positive up-regulation of p53 and contribution to its documented accumulation. Up-regulated p21 supports enhanced transcriptional activity for genes involved in cell cycle arrest. Importantly, these data also point to p53 induced p21 up-regulation in vitiligo. Our MDM4 data suggest selective tissue damage-dependent activity. This later assumption requires more work.

Up-regulated epidermal SPARC expression – a radical scavenger in vitiligo skin? A novel result.

Highly expressed matrix cellular protein SPARC is an important marker for melanoma aggressiveness via facilitating invasion and metastatic behaviour of melanoma cells (Ledda et al., 1997; Sturm et al., 1997; Massi et al., 1999; Alvarez et al., 2005; Alonso et al., 2007). Recently Fenouille and colleagues suggested a novel role for SPARC in melanoma. These authors reported that melanoma cells with high SPARC expression are characterised by low levels of p53. Moreover, these authors showed induction of G2/M cell cycle arrest and promotion of p53-dependent p21 up-regulation via SPARC depletion. In the same year this group also introduced a role for SPARC in triggering AKT-dependent phosphorylation of MDM2, leading in turn to its stabilization, followed by degradation of p53 (Fenouille et al., 2011a;b).

In our context, we decided to examine expression of SPARC in vitiligo to get a better understanding of the possible mechanism behind of up-regulated functioning wild type p53 in association with no increased risk of solar induced non-melanoma skin cancer, younger-appearing skin compared to age matched healthy persons (Calanchini- Postizzi and Frenk, 1987; Schallreuter et al., 2002; Salem et al., 2009; Teulings et al., 2013; Schallreuter, 2014) but with a possible significantly higher risk for melanoma (Schallreuter et al., 1991a).

Our *in situ* immuno-fluorescence labelling of SPARC as well as Western blot investigations identified for the first time up-regulated SPARC in the entire epidermis in lesional and non-lesional skin of classic vitiligo **(Figures 43, 45).** Therefore, our data support the reported increase in SPARC expression in kerationcytes in response to different cellular stresses including the exposure to sodium arsenite (Kudo et al., 1994) which later on was documented as an oxidative stress / DNA damage-inducing agent in breast cancer cells via induction od ROS production (Ruiz-Ramos et al., 2009). Given, that oxidative / nitrative stress is present in vitiligo, it is tempting to conclude that up-regulation of SPARC in vitiligo is very likely the consequence of cellular stress. Expression was also high in melanocytes of non-lesional skin **(Figure 47)**. Our *in vitro* studies on epidermal melanocytes from healthy individuals confirm SPARC expression as shown by immuno-fluorescence and Western blot (**Figures 48, 49).** These results are supported by a publication from Robert and colleagues (Robert et al., 2006). However, it is also of note, that two other publications reported absence of SPARC in normal epidermal melanocytes (Ledda et al., 1997 and Kato et al., 2000).

Computer simulation supports loss of functional SPARC in classical vitiligo via oxidative and nitrative stress suggesting SPARC up-regulation as ROS scavenger.

Given that, patients with vitiligo accumulate functioning wild type p53 in the presence of high H_2O_2 / ONOO- levels, we proposed the possibility of deactivation of epidermal SPARC by this ROS / RNS. One expected consequence would be prevention of $p90^{MDM2}$ - phosphorylation by a dysfunctioning protein, leading in turn to loss of control on p53 up-regulation and p53 functionality.

To further elucidate this scenario, we utilised computer simulation to follow the effect of nitration and oxidation by H_2O_2 / $ONOO^-$ on the protein structure of SPARC. As this protein is calcium dependent, containing two high affinity EF-hands calcium-binding sites, located at the α-helical domain (EC domain, residues 138–286) (Maurer et al., 1995; Hohenester et al., 1996; Kretsinger, 1996, Hohenester et al., 1997), we investigated the effect of oxidative / nitrative stress specifically on SPARC-calcium binding capability. Co-localization of SPARC and 5-nitro-tyrosine revealed massive nitration of SPARC protein throughout the entire epidermis in vitiligo skin with more pronounced localization in cellular walls and some nuclei **(Figure 50).**

The results of computer simulation further support $ONOO^-$-mediated nitration of SPARC in vitiligo, as already indicated by the double immuno-fluorescence data. Moreover, SPARC is also considerably affected by H_2O_2-mediated oxidation and combined oxidation / nitration by H_2O_2/ $ONOO^-$ on both calcium EF-hands binding sites. These effects lead to a major loss of O - donor atoms in both binding sites of native SPARC with a likely loss of calcium. Both observations, together with significantly up- regulated p53 / $p76^{MDM2}$ / p21 in the entire epidermal compartment of patients with vitiligo, in both lesional and non-lesional skin, suggest SPARC inactivation with loss of functionality via $ONOO^-$ / H_2O_2- mediated stress as one general event in vitiligo.

SPARC has been implied in triggering tumour development of melanoma in a p53-dependent manner (Fenouille et al., 2011a;b). Given, that suppression of SPARC would activate p53 / p21 - enhanced cell cycle arrest, leading in turn to inhibition of cell proliferation (Fenouille et al., 2011), then it is tempting to propose that these structural changes of SPARC, accompanied with its loss of wild type p53 regulation in response

to H_2O_2 / $ONOO^-$ in vitiligo, may lead to protection of melanoma development in our patients investigated in our study.

However, our results are in sharp conflict with Fenouille's work, who proposed a fundamental SPARC / p53 regulation in melanoma (Fenouille et al., 2011 a; b). Clearly future work is needed to shed some more light on this scenario.

Is up-regulated VEGF-A expression enhancing SPARC in vitiligo?

Earlier it was reported that VEGF-A induces SPARC expression in human vascular endothelial cells (Kato et al., 2001; Weninger, et al., 1996). Induced SPARC may trigger a negative regulatory feedback mechanism via binding to VEGF-A as a consequence prevent activation of VEGFR1 (Kupprion et al. 1998; Nozaki et al. 2006).

In the context of our aim, it is of note that VEGF-A is present in keratinocytes and that this expression can be enhanced by NO and H_2O_2 (Brauchle, et al., 1996; Frank et al., 1999). Hence, this scenario is a valid condition in vitiligo. We expected up-regulated epidermal VEGF-A in this disease. And this was indeed the case, as we found significantly higher levels throughout the entire epidermis in lesional and non-lesional skin of our patients compared to controls **(Figures 57, 59).**

Immuno-fluorescence and Western blot showed undetectable VEGF-A levels in control melanocytes **(Figures 61, 62),** while expression is high in melanocytes of non-lesional skin **(Figure 60).**

Based on the fact that keratinocytes show significantly increased VEGF-A expression during wound healing, e.g.in psoriasis and in response to UV- irradiation (Brown, et al., 1992; Detmar, et al., 1994; Detmar, et al., 1995; Brauchle, et al., 1996; Weninger , et al., 1996; Mildner, et al., 1999; Gille, et al., 2000; Lauer, et al., 2000), an important role has been proposed for VEGF-A in regulation of vessel function under physiological

conditions (Weninger et al., 1996). In this context, the presence of increased VEGF-A positive cells as well as VEGF-A positive vessels were recently described in the centre of vitiligo lesions but not in the periphery (Aroni et al., 2010).

The increased vascularisation was proposed as the end result of active immune mechanisms as part of a healing / repair process in vitiligo (Aroni et al., 2010). Although these authors expected correlation between VEGF-A-induced vascularisation and increased lymphocytes, in the end they failed to prove their concept (Aroni et al., 2010).

At the same token we cannot ignore the critical role of VEGF-A in induction of angiogenesis in malignant melanoma (Claffey et al., 1996) by stimulating proliferation, migration, invasion, anti-apoptotic function and increasing vascular permeability (Alon et al., 1995; Dvorak et al., 1995). However, in our vitiligo cases, studied herein, no signs of skin cancer has been detected so far, despite of increased VEGF-A expression in melanocytes and keratinocytes. These data suggest that VEGF-A could drive benign vascularisation in vitiligo.

Based on our data, it is tempting to speculate that increased SPARC expression / levels in vitiligo serve as "antioxidant" in response to oxidative / nitrative stress after induction via VEGF-A. To our knowledge this would be a completely new function for SPARC. This assumption is supported by computer simulation, showing that VEGF-A is only little affected by H_2O_2 - mediated oxidation and $ONOO^-$ - mediated nitration (personal communication with Dr NCJ Gibbons), data not shown.

Evidence for H_2O_2 - and $ONOO^-$ - mediated deactivtion of TGF-β1 in vitiligo supports p53 dependent p21 induction.

As a regulatory role for TGF-β1 has been documented in up-regulation of p21 expression in a p53-independent manner (Rodeck et al., 1994; Krasagakis et al., 1999; Rodeck et al., 1999; Hoek et al., 2006) and SPARC induces up-regulation of TGF-β1, we finally included evaluation of TGF-β1 in our study. Our *in situ* immuno-fluorescence results show significantly up-regulated TGF-β1 expression in both, lesional and non-lesional skin of our patients, with a distinct stronger expression in the basal and suprabasal cells **(Figures 63, 64)**. Interestingly, only melanocytes of non-lesional skin show strong TGF-β1 expression **(Figure 65)** which is completely absent in one detached lesional melanocyte and in healthy controls under *in situ and in vitro* condition **(Figures 65, 66).** Based on our results that SPARC is significantly up-regulated, but not functioning after H_2O_2 – mediated oxidation and $ONOO^-$-mediated nitration, it is tempting to conclude that TGF-β1 up-regulation in vitiligo escapes regulation by SPARC. Hence, the question to be answered is, whether up-regulation of TGF-β1 is under direct control in response to oxidative / nitrative stress. This assumption would be supported by increased TGF-β1 in human diploid fibroblasts after exposure to UVB via inducing H_2O_2 (Frippiat et al., 2001; Debacq-Chainiaux et al., 2005). Given, that TGF-β1 is nitrated as shown by double immuno-fluorescence **(Figure 68),** then one must consider that up-regulation of TGF-β1 is very likely a SPARC independent event in vitiligo. As functioning TGF-β1 up-regulates p21 expression, it is tempting to conclude that the observed up-regulated p21 in vitiligo is a consequence of up-regulated p53 and not controlled by TGF-β1. The data from the computer simulation support this conclusion (personal discussion with Dr CJ Gibbons).

In summary, confirmation of the previous data, together with our new data presented in this thesis, further support a central role for up-regulated functioning wild type p53 as a major player in the pathogenesis of vitiligo. One other very important result stems from this study.Our data further substantiate that in classical vitiligo the entire epidermal unit is part of the disease process and not only the epidermal melanocyte.

Part II

Comparison of MAL and classical vitiligo - a case control study

6. Introduction

6.1 Malignant Melanoma (MM) in association with "vitiligo-like" leucoderma

Skin cancer is the most common malignancy in fair skinned people worldwide (Breitbart et al., 2006). It can be classified into malignant melanoma (MM) and non-melanoma skin cancers (NMSC). NMSC involves basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and the precursor i.e. actinic keratoses (AC). UVR is considered to be the main risk factor for all of them. These types of skin cancer arise in the majority of sun-exposed areas in fair-skinned people. Moreover, the incidence is negatively proportioned to skin colour and positively to the amount of exposure to ultra violet radiation (Preston and Stern, 1992; Diepgen and Mahler, 2002; Suarez et al., 2007). Melanoma is a malignant tumor arising from melanocytes. It predominantly occurs in skin (95%), but is also found in other parts of the body, where melanocytes are present including eye, mucous membranes, gastrointestinal tract, genitourinary tract and leptomeninges.(Markovic et al., 2007). Worldwide manifestation of melanoma is increasing faster than any other malignancies. It represents less than 5% of all skin cancers, but is the cause of approximately all skin cancer's deaths (Rigel, 2008). Hence, melanoma represents a major clinical problem, as once the disease is spreading beyond loco-regional sites, there are currently no curative treatments (Soengas and Lowe, 2003; Tsao et al., 2004; Thompson et. al., 2005). New data from the United States documented 76.000 new cases with melanoma and over 9.000 deaths for the year 2013. The rise of incidence was around 6% per annum in the 1970's. At the current time this increase is rising over 3% per annum (Naveh et al., 2013).

Malignant transformation of melanocytes to metastatic melanoma and its escape from keratinocyte-controlled regulation takes place via a group of carcinogenic events such as mutations in some genes that are involved in the regulation of cell growth, production of autocrine growth factors as well as melanocytes adhesion receptors loss (Haass et al., 2004). This transformation passes five main steps:

1. Proliferation and spread of cytological normal melanocytes forming a benign nevus cell nevus.

2. These nevi could develop aberrantly forming a dysplastic cell nevus with random structural atypia. Dysplastic nevi are considered as premalignant lesions.

3. Radial growth phase (RGP) in primary melanoma with unlimited hyperplasia may develop leading in turn to dermal micro-invasion.

4. Formation of vertical growth phase (VGP) melanoma.

5. Those VGP cells may continue in uncontrolled growth, with more invasion efficiency and these cells can invade other parts of the body including lung, liver, brain and bone via the blood stream. **(Figure 69).**

Not all melanomas exhibit these five steps because some RGP or VGP can arise from melanocytes directly without the presence of pre-existing nevi (Miller and Mihm, 2006). Different steps of melanoma development were found to be associated with a group of molecular changes leading in turn to unlimited proliferation, invasion and metastasis of melanoma cells. Some of these changes are summarised in **(Figure 69)** (Miller and Mihm, 2006).

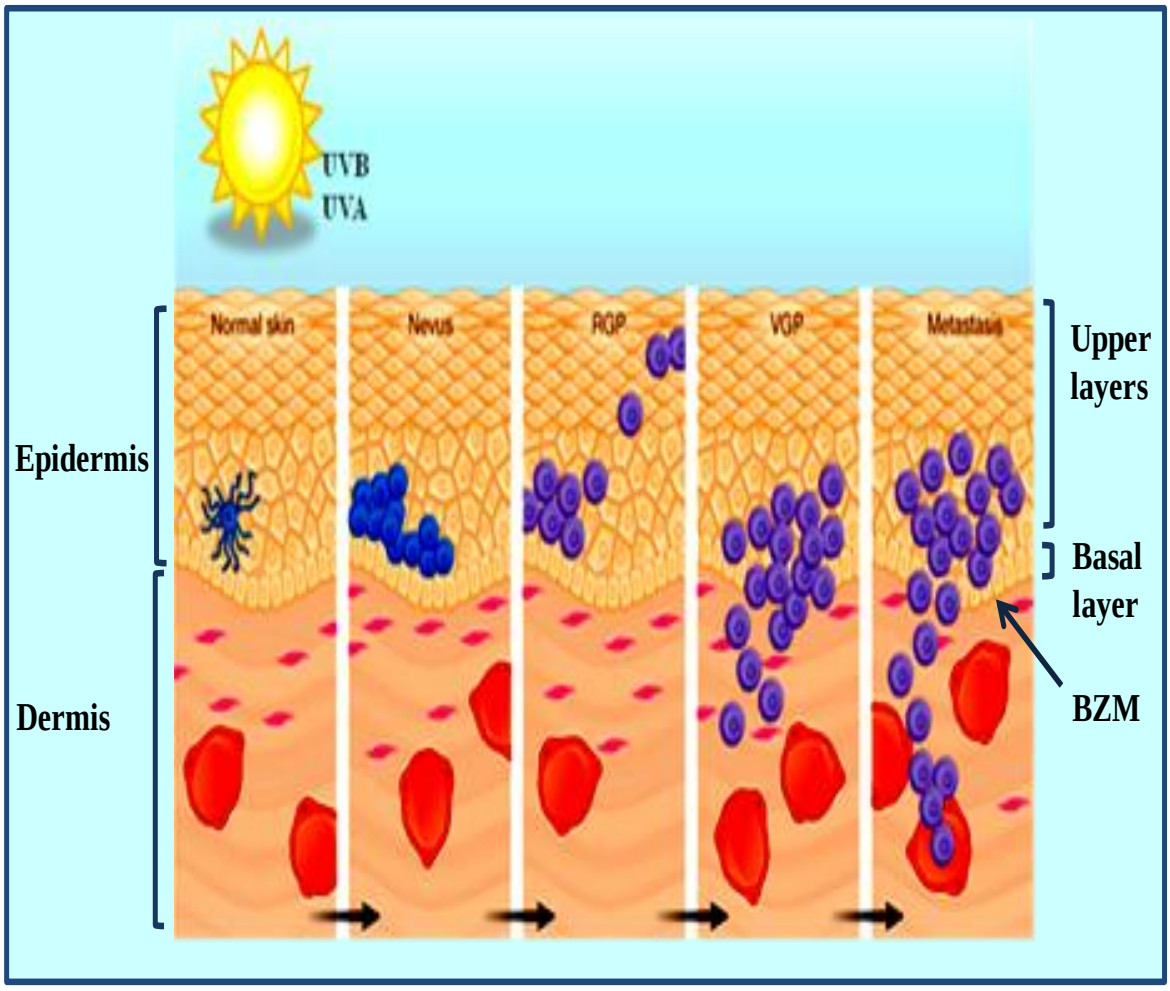

Figure 69: Proposed molecular alterations associated with the initiation and progression of melanoma.

This illustration depicts human melanoma progression. Aberrant proliferation of normal melanocytes, presumably in response to UV radiation, results in the formation of benign or dysplastic nevi. Radial growth phase (RGP) melanoma exhibits the ability to grow intra-epidermal, followed by invasion of the dermis in the vertical growth phase (VGP), and culminating with metastasis. Note that only about half of the melanomas are known to arise from nevi, and progression can occur without going through all the stages depicted. Several melanoma susceptibility genes have been identified in kindred and have been validated in mouse models **(modified from Zaidi et al., 2008).**

Over recent decades the post surgical appearance of white patches in patients with melanoma has received much attention. This phenomenon is described with various names, including vitiligo, melanoma-associated vitiligo, melanoma associated hypomelanosis, melanoma associated depigmentation, melanoma associated leucoderma (MAL) and vitiligo-like depigmentation (Nordlund et al., 1983; Ram and Shoenfeld, 2007; Hartmann et al., 2008; Teulings et al., 2015). Even to date, it is obvious that there is no common consensus so far, concerning this clinical entity.

Several types of leukoderma have been described in association with melanoma.

- **Primary melanoma regression** is a progressive process, replacing the tumour with fibrous stroma within the superficial dermis, although complete regression of melanoma is rare (Naveh et al., 2013).
- **Halo nevus (Sutton's nevus)** presents a white rim around a pigmented nevus. It is frequently associated with vitiligo and melanoma (Bolognia 2009; Naveh et al., 2013).
- **Melanoma-associated depigmentation (leucoderma)** describes a post surgical appearance of white patches in sites, distant from the primary tumour, arising either spontaneously or following immunological based treatments (Naveh et al., 2013; Teulings et al., 2015).

In our study we will designate this special post surgical depigmentation as **melanoma associated leucoderma (MAL)**, originating from leucos / white and derma / skin as we feel that this is the best description.

6.2 Vitiligo and malignant melanoma- Is there a true connection?

Briefly, vitiligo is a depigmenting skin disorder with varying clinical presentations classified to localized (segmental, focal) and generalized (acro-facial, vitiligo vulgaris and universal or mixed). The pathogenesis is still not completely understood despite many past and recent efforts (Schallreuter, 2014). Although vitiligo is an old disease, it was recently re-classified by the European Task Force group and an international consensus group in non-segmental, segmental, mucosal and mixed vitiligo (Taïeb and Picardo, 2007; Ezzedine et al., 2012). However, interestingly, melanoma-associated leucoderma (MAL) is not included as subtype of vitiligo.

In 1991 Schallreuter and colleagues proposed an increased risk for development of cutaneous melanoma in patients with vitiligo (Schallreuter et al., 1991a). Those data were based on a group of 623 patients with melanoma. Eleven of them were developed depigmentation more than 2 years prior to diagnosis of the primary tumour (n=11/623). A second group of patients (n=11/623) developed depigmentation post surgical excision of the primary melanoma. Whether this latter depigmentation (leucoderma) is a classical vitiligo or not, is still under debate. A recent study based on a postal survey included 2635 patients with non-segmental vitiligo, > 50 years at the time of the study. Fifty % eligible questionnaires were returned (n=1307). The time period ranged from 1995-2010. Partners of patients served as controls. All skin cancers were validated by a pathology report. Multivariate logistic regression models were used to quantify adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for associations between vitiligo and lifetime prevalence of MM and NMSC. According to this study, patients with vitiligo had a 3-fold lower probability of developing melanoma (Teulings et al., 2013). As only 50% of the questionnaires were returned several possibilities cannot be ruled out. The

number of patients, who did not answer the questionnaire, could have had melanoma and passed already away. Moreover, the patient age (>50years) would have missed all melanomas in younger patients. Hence, more thorough studies are needed to answer the question, whether there is indeed a higher risk for patients with classical vitiligo to develop melanoma. A study published in 2012, investigating the incidence of melanoma from 1970 to 2009 showed an 8-fold increase among young women and 4-fold among young men, who developed their melanoma between the age of 18 to 39 years (Reed et al., 2012).

6.3 MAL in association with malignant melanoma – Is it a good prognostic factor?

In the context of the clinical outcome of melanoma, MAL has been reported and proposed as a favourable prognostic factor for tumour survival in this patient group with significantly enhanced 5-year survival rates (Nordlund et al, 1983; Bystryn et al, 1987; Quaglino et al, 2010). Development of depigmentation after immunotherapy has been correlated with improved survival rates (Rosenberg and White, 1996; Boasberg et al, 2006; Gogas et al, 2006). As said above, a better overall outcome has been proposed for patients with melanoma, who develop vitiligo / depigmentation after excision of the tumour or after immunotherapy. A very recent publication systemically evaluated 137 studies published between 1995 and 2013 on melanoma immunotherapy of stage III and stage IV melanoma that reported on autoimmune toxicity and / or vitiligo. Methodological quality of each study was based on adapted criteria for systematic reviews in prognostic studies. Random-effect models were used to calculate summary estimates of the cumulative incidence of vitiligo-like depigmentation across all studies.

The prognostic value of this depigmentation on survival outcome was assessed, using random-effects Cox regression survival analyses. In 27 studies, reporting individual patient data, vitiligo development was significantly associated with both, progression-free-survival (hazard ratio [HR], 0.51; 95% CI, 0.32 to 0.82; p< .005) and overall survival (HR, 0.25; 95% CI, 0.10 to 0.61; $p < .003$), indicating that these patients have a 2-4 times lower risk of disease progression and death, respectively, compared to patients without development of vitiligo (Teulings et al., 2015). The use of vitiligo and vitiligo-like depigmentation in this article points once more to the necessity for more fundamental knowledge of this tumour associated depigmentation.

Two important questions came to our mind. Why did these patients develop post - surgical leucoderma? The other intriguing question to be answered is, whether MAL is actually the same entity as classical vitiligo.

6.4 p53 and malignant melanoma

Inactivation of p53 is considered to be one of cancer characteristics, as 80% of tumours have p53 missense mutations and production of aberrant proteins with abnormal functions leading to tumour progression and metastasis (Lozano, 2007). In contrast to these types of tumours, melanomas mostly hold wild type p53. Mutation of p53 in patients with melanoma is ranging from 0 to 30% (Albino et.al., 1994; Papp et al., 1996; Zerp et al., 1999; Ragnarsson-Olding et al., 2002; Daniotti et. al., 2004; Houben et al., 2011). So far all mutations identified, lead to transcriptional inactivity of p53 protein (Houben et al., 2011). p53 is predominantly highly expressed with nuclear localization. Its expression correlates with tumour progression and invasion. High p53 expression is considered as a bad prognostic sign (Yamamoto and Takahashi, 1993; Montano et al., 1994; Sparrow et al., 1995; Zerp et al., 1999; Rass et al., 2001; Soussi and Beroud, 2001;

Soto et al., 2005; Gwosdz et al., 2006; Li et al., 2006). Despite the presence of high p53 expression levels in melanoma, a recent study showed a significant alteration in expression of several p53 target genes, involved in apoptosis and cell cycle regulation (e.g. p21) (Avery-Kiejda et al., 2011). Moreover, inhibition of p53 in melanoma cell lines had limited effect on p53 target gene expression and resulted in decreased proliferation of melanoma cells. This result could suggest, that p53 lost the ability to regulate its target genes. Rather than acting as a tumour suppressor, it may promote melanoma proliferation and progression (Avery-Kiejda et al., 2011).

6.5 p53 / MDM4 interaction in malignant melanoma

Recently Gembarska and colleagues reported that MDM4 is over-expressed in about 65% of melanoma (Gembarska et al., 2012). Taking into consideration that p53 mutations are rare in melanoma, these authors proposed that MDM4 over-expression is an important oncogenic event that alters p53-function in a large proportion of patients. Moreover, it was suggested that melanocyte MDM4 over-expression coincides with NRAS oncoprotein, resulting in turn in a shorter time of melanoma development and more aggressive melanoma formation. Hence, it was concluded that targeting the MDM4 / p53-interaction should lead to growth inhibition of melanoma. These data demonstrated that high MDM4- expression levels are essential for proliferation of melanoma cells in culture, in a p53-independent manner (Gembarska et al., 2012).

6.6 p53 / SPARC interaction in malignant melanoma

In some types of malignancies SPARC exerts tumour suppression function, as it is inactivated or expressed in low levels, leading to aggressive and metastatic behaviour e.g. in colorectal cancer (Yang et al., 2007; Cheetham et al., 2008), in pancreatic cancer (Puolakkainen et al., 2004) in ovarian cancer (Yiu et al., 2001) and in acute myeloid leukemia (DiMartino et al., 2006), while in other types of cancer SPARC seems to have a crucial role in promoting tumour progression, as it is expressed in very high levels in these tumours e. g. breast cancer (Bellahcene and Castronovo, 1995; Porter et al., 1995; Jones et al., 2004; Lien et al., 2007), glioblastomas (Rempel et al., 1998) and melanoma (Ledda et al., 1997).

During progression of malignant melanoma, an alteration in the expression of cell–matrix and cell–cell communication molecules, including SPARC, was documented (Haass et al., 2005b; Miller and Mihm, 2006). It was found that SPARC is strongly expressed by advanced primary and metastatic melanomas in both neoplastic cells as well as melanoma associated stromal cells with a weaker expression in nevus cells, while its expression is absent in normal melanocytes (Ledda et al., 1997; Kato et al., 2000; Wong and Rustgi, 2013). Fenouille and colleagues reported in 2011 that depletion of SPARC in melanoma cells yields activation of p53 and induction of p21, leading in turn to G2/M cell cycle arrest and tumour growth inhibition (Fenouille et al., 2011a;b).

6.7 SPARC / TGF-β1 / VEGF-A cascade in malignant melanoma

Increased expression of TGF β 1, β 2 and β 3 proteins has been described in invasive primary melanomas as well as in metastatic nodules (Van et al., 1996). Moreover, TGF-β1 is playing a crucial role in modulating the stroma surrounding melanoma cells through paracrine activity (Berking et al., 2001). TGF-β1 increases the expression of SPARC protein as well as its mRNA (Nakamura et al., 1996; Damjanovski et al., 1998). SPARC also induces the expression and secretion of TGF-β1 suggesting in turn a reciprocal regulatory feedback loop between SPARC and TGF-β1 (Bassuk et al., 2000). Moreover, TGF- β1 enhances p21 expression through p53-independent mechanism (Rodeck et al., 1994; Krasagakis et al., 1999; Rodeck et al., 1999; Hoek et al., 2006).

VEGF-A is detected in keratinocytes in response to NO and H_2O_2 (Brauchle, et al., 1996; Frank et al., 1999). Malignant melanoma cells also express VEGF-A to induce the formation of new blood vessels (angiogenesis) (Claffey et al., 1996) via stimulating proliferation, migration, invasion, anti-apoptotic function and permeability of vascular endothelial cells (Alon et al.,1995; Dvorak et al.,1995). VEGF-A induces the expression of SPARC in human vascular endothelial cells (Kato et al., 2001; Weninger, et al., 1996). This induction of SPARC might be a negative regulatory feedback mechanism as SPARC binds VEGF and prevents activation of VEGFR1 (Kupprion et al., 1998; Nozaki et al., 2006).

7. Aim of the study (part II)

Given, our long standing basic and clinical knowledge in the field of vitiligo, it was the aim of this study to use *in vivo* and *in vitro* observations for comparison and characterisation of melanoma associated leucoderma (MAL) and classical vitiligo.

For this purpose we decided to use several of those results as applied and shown in Part I of this thesis for investigation of MAL, utilising immuno-fluorescence, Western blot and *in vivo* FT-Raman spectroscopy. Considering that patients with classical vitiligo do present a partial loss of their inherited protective skin colour, but without an increased risk for solar induced skin cancer or increased photo-damage in association with up-regulated functioning wild type p53 and DNA-damage together with an efficient DNA-repair machinery (Schallreuter et al., 2003; Salem et al., 2009), two open crucial questions remained to be answered in the case of MAL and / or classical vitiligo.

1. Is development of postsurgical and / or immune-induced leucoderma indeed classical vitiligo?
2. Is development of postsurgical / immune-induced leucoderma a good prognostic factor for melanoma survival rates?

Bearing those questions in mind, we felt, it was only timely to compare those data originating from classical vitiligo with post surgical melanoma associated leucoderma (synonyms used: vitiligo, vitiligo-like leucoderma).

As epidermal low or absent epidermal catalase expression is one characteristic marker for classical vitiligo, correlating with epidermal accumulation of 10^{-3}M H_2O_2 levels, as documented by *in vivo* FT-Raman spectroscopy, we wished to utilise this scenario in our study of MAL. In this context it is of note that a

constantly up-regulated functioning p53 in classical vitiligo has been implied as the major contributor to DNA-repair and prevention of increased solar induced skin cancer. Hence, we decided to follow p53 and p21 together with the MDM2 family and regulation by SPARC in MAL (Schallreuter et al., 2003, Salem et al., 2009, Schallreuter 2014). Moreover, as both VEGF A and TGF-β 1 regulate p53 and SPARC expression as well as p21 in a p53-independent manner can foster hypopigmentation in cultured melanocytes and melanoma cells, we wished to include these signals in our study (Martínez-Esparza et al., 1997; Martínez-Esparza et al., 2001; Kim et al., 2003).

8. Materials and methods

8.1 Patient history and characteristics of our MAL subject

The study was in agreement with the local Ethics Committee of the EM Arndt University of Greifswald / Germany and with declaration of Helsinki principles. A written consent was obtained from the patient.

The patient developed a cutaneous nodular ulcerated malignant melanoma in a congenital naevus at his left upper extremity in 9/2008. The tumour was excised without lympho nodectomy. Histology revealed a predominant epitheliod cell type. Breslow level of 4.75 mm and Clark level 4. Absence of microsatellites and tumour infiltrating lymphocytes. No signs for perineural and vascular invasion. No tumour spreading until 3/2015. Development of symmetrical depigmentation 6 months post surgery in the face, chest, upper extremities including palms and lower extremities. No signs of depigmentation / regression in the congenital naevus prior to excision. The clinical examination in 9/2008 showed no halo naevi. Wood's light examination revealed a weak yellowish fluorescence pointing to oxidised pterins as in classical described for classical vitiligo (Schallreuter et a., 1994a). No other immune diseases present. Negative family history for vitiligo and melanoma. Otherwise the patient was in good general health. Laboratory evaluation at 9/2011 shows low vitamine D levels, low vitamine B12 levels, borderline cholesterol elevation, otherwise unremarkable results.

Figure 70: Facial MAL under WOOD's light in a 53 year old patient with melanoma (skin phototype III, Fitzpatrick classification) (With kind permission from Professor Dr KU Schallreuter).

8.2 In *vivo* Fourier Transform (FT) - Raman spectroscopy

FT-Raman spectra were acquired using a BRUKER RFS 100/S spectrometer (Bruker, Karlsruhe, Germany) with a liquid-nitrogen-cooled Germanium detector. Near-infrared excitation was produced by a Nd^{3+}:YAG laser operating at 1064 nm. Each spectrum was accumulated over 17 min with 1000 scans and a resolution of 4 cm^{-1}. Detection of H_2O_2 is based on the O=O stretch at 875 cm^{-1}. The Met-S=O-stretch was visualised at 1030 cm^{-1} accordingly. L-tryptophan and its oxidation products 5-OH-tryptophan (930 cm^{-1}) and N-formyl-kynurenine/kynurenine (1050 cm^{-1}) were recently assigned. This experiment was done in collaboration with Dr H. Rokos.

8.3 Full skin biopsies from lesional and non-lesional skin

After written consent total skin biopsies were taken under local anaesthesia from lesional and non-lesional skin of the right volar forearm, when the patient was seeking help in the Institute for Pigmentary Disorders in Greifswald / Germany for the rapid loss of his facial skin colour. In addition we used 3 healthy controls with skin photo type III (Fitzpatrick-classification). Biopsies were taken under the same conditions from controls from the volar forearm.

8.4 Preparation of cryosections

MAL and control skin biopsies were embedded in optimal cutting temperature compound (OCT) (Sakura, RA Lamb, Eastbourne, UK) and placed for about 5 minutes in liquid N2 to prevent any post surgical detoriation of the material. Sections were cut (3-5 μm) using a Leica CM3050 S cryostat (Leica Microsystems, Milton Keynes, UK) and placed onto commercially available poly-L-lysine coated slides (Sigma, Pool, Dorset, UK). The slides were saved at -80°C for future work

8.5 *In situ* immuno-fluorescence labelling

Frozen slides were allowed to defrost at RT for 10 minutes followed by dehydration in 1 x PBS for 5 minutes. Fixation was performed by immersing slides for 6 minutes in ice cold- methanol. Then slides were washed in 1 x PBS for 5 minutes, followed by blocking with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) for 90 minutes at RT. Slides were washed once in 1 x PBS for 5 min followed by another wash once in Tween-20 (0.05%) for 5 minutes and then twice again in 1 x PBS for 5 minutes each. Afterward slides were incubated overnight at 4°C with the primary antibodies, diluted in 1% NDS. Thereafter, slides were washed once in 1 x PBS for 5 minutes, followed by another wash once in Tween-20 (0.05%) for 5 minutes and then twice again in 1 x PBS for 5 minutes each. After that, slides were incubated for 1 hour at RT with a fluorescent secondary antibody in a dilution of 1:50 in 1% NDS for 1 hour (donkey anti-rabbit or mouse depending on the primary antibody (Jackson Immuno Research Laboratories, Cambridgeshire, UK). Slides were washed once in 1 x PBS for 5 minutes, then once in Tween-20 for 5 minutes followed by two washes in 1 x PBS for 5 minutes each. Finally, slides were dried and mounted using Vectashield Mounting Medium containing 4, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) and covered with a coverslip. Pictures were viewed with a Leica DMIRB/E fluorescence microscope (Wetzlar, Germany). Images were captured using a Nikon Eclipse 80i microscope with a DS-U101 Nikon camera coupled to a Nikon ACT-2U capture program (Nikon, Europe). The antibodies and dilutions used are listed in (**Table 6).** Negative control staining was carried out by omitting the primary antibody from the staining procedure and substitution of 1% NDS / PBS.

8.6 Double immuno-fluorescence labelling

In situ double immuno-fluorescence was used to detect co-localization of two specific proteins within one section. The protocol was similar to the described mono staining with some small modifications. After incubating skin sections with the 1st secondary antibody and a brief rinse in PBS, sections were blocked with normal donkey serum (10% NDS, Jackson Immunoresearch Laboratories, Cambridgeshire, UK) for 90 minutes at RT. Slides were washed once in 1 x PBS for 5 minutes, followed by two washes, once in Tween-20 (0.05%) for 5 minutes and then twice again in 1 x PBS for 5 minutes each. Slides were incubated overnight at 4°C with the 2nd primary antibodies, diluted in 1% NDS. The rest of the labelling was followed as described in detail. ImageJ version 1.37 was utilised (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html) to merge FITC- and TRITC- images for evaluation of co-localization. The antibodies and dilutions used are listed in (**Table 6).**

8.7 Quantification of fluorescence intensity

To quantify the fluorescence, ImageJ version 1.37 was utilised (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). The region to be quantified was highlighted and a mean intensity obtained. This was repeated for the rest of the layers. The procedure was repeated in at least three epidermal sections of each patient and the mean value was obtained and plotted using Microsoft Excel (Microsoft Corporation, USA). Significance of data was analysed statistically using two-tailed unpaired t-test.

Antibody	Dilution	Incubation time/T	Source	Company	Cat. No.
catalase	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16731
$p76^{MDM2}$	1:20	Overnight/ 4°C	Mouse monoclonal antibody	Santa cruz biotechnology	sc-965
MDM4	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16058
MDM4-phospho	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab70236
Nitrotyrosine	1:50	Overnight/ 4°C	Mouse monoclonal antibody	Abcam, Cambridge, UK	ab78163
NKI/beteb	1:50	Overnight/ 4°C	Mouse monoclonal antibody	Caltag-Medsystems Ltd	MON7006-1
p21	1:5	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab7960
p53	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab1431
SPARC	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab14174
TGF-β1	1:50	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab66043
VEGF-A	1:100	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab46154

Table 6

List of primary antibodies and conditions for *in situ* immuno-fluorescence labelling.

8.8 Western blot

In order to perform Western blotting, the protein samples were first resolved in SDS PAGE gel electrophoresis as described before. After activation of polyvinylidene difluoride (PVDF) membrane (GE Healthcare, formerly Amersham Biosciences) by immersing it in methanol for about 2 minutes, the gel was sandwiched (1 sponge (cathode), 1 filter paper, gel, PVDF membrane, 1 filter paper, 1sponge (anode)) and placed in the electro blotting chamber filled with transfer buffer pH 8.3 (3g Tris Base, 14.4g glycine, 0.4g SDS, 1000 ml distilled H_2O). The transfer was run at 30 Volts for about 3 hours. Afterwards the PVDF membrane was immersed in non-fat milk (5%) (2.5g non-fat milk, 50 ml TBS / Tween(8.28g NaCl, 2.42g Tris, 0.47ml Tween 20, make volume to 1000ml using distilled water)) for 2 hours at room RT to block nonspecific binding sites. The membrane was incubated overnight at at 4^oC with primary antibody. The antibodies and used dilutions are listed in **Table 7.** All the previous antibodies were diluted in 5% w/v non-fat dry milk powder in TBS / Tween. This was followed by 3 washing steps within 30 minutes in TBS / Tween under continuous shaking. The membrane was transferred into a plastic dish containing one of secondary antibodies **(Table 8)** which was diluted in 5% w/v non-fat dry milk powder in TBS / Tween depending on the host of the primary antibody and incubated for 1 hour at RT, followed by wash in TB / Tween. Positive immune-reactivity was detected by the enhanced chemiluminescence's method (ECL). To do so, the membrane was immersed for 4 minutes in equal volumes of enhanced chemiluminescence's solution I (1 ml of 250 mM luminol in DMSO, 0.44 ml of 90 mM p-coumaric acid in DMSO, 10 ml of 1 M Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma, Pool, Dorset, UK) and solution Π (64 µl of 30% v/v H_2O_2, 10 ml of Tris-HCl pH 8.5 in a final volume of 100 ml) (Sigma,

Pool, Dorset, UK). Positively stained protein bands send a luminescent signal which was visualised on a sheet of CL-XPosure Film (Thermo scientific, UK).

Antibody	**Dilution**	**Incubation time/T**	**Source**	**Company**	**Cat. No.**
SPARC	1:500	Overnight at 4^{o}C	Mouse monoclonal	Santa cruz biotechnology	sc-73051
p53	1:500	Overnight at 4^{o}C	Rabbit polyclonal	Santa cruz biotechnology	sc-6243
GAPDH	1:5000	Overnight at 4^{o}C	Rabbit polyclonal	Abcam Cambridge, UK	ab9485
GAPDH	1:5000	Overnight at 4^{o}C	mouse monoclonal	Abcam Cambridge, UK	ab8245
p21	1:500	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab7960
TGFβ1	1:2000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab66043
MDM2	1:300	Overnight/ 4°C	Mouse monoclonal antibody	Santa cruz biotechnology	sc-965
MDM4	1:1000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab16058
MDM4-phospho	1:	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab70236
VEGF-A	1:1000	Overnight/ 4°C	Rabbit polyclonal antibody	Abcam, Cambridge, UK	ab46154

Table 7

List of primary antibodies used in Western blot in MAL.

Antibody	Dilution	Incubation time	Company	Cataloge number
Anti-Mouse IgG Peroxidase antibody produced in goat	1:1000	1h/RT	Sigma-Aldrich, St. Louis, USA	A2554-1ML
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	1:1000	1h/RT	Sigma-Aldrich, St. Louis, USA	A9169-2ML

Table 8

List of Secondary antibodies used in Western blot

8.9 Statistical analysis for Western blot

The bands were evaluated by utilizing Image J version 1.37 (supplied online by NIH at http://rsb.info.nih.gov/ij/index.html). Each band was highlighted and the intensity was calculated. Statistical analysis was carried out by Microsoft Excel. The mean of the calculated values were plotted in the figures.

9. Results

9.1 *In vivo* FT - Raman analysis identifies the presence of H_2O_2 - mediated oxidation in lesional and non-lesional MAL.

In vivo FT-Raman spectroscopy detects H_2O_2 levels in the 10^{-3}M range in the skin of the right volar inner forearm of MAL. This result is not different compared to classical vitiligo **(Figure 71).**

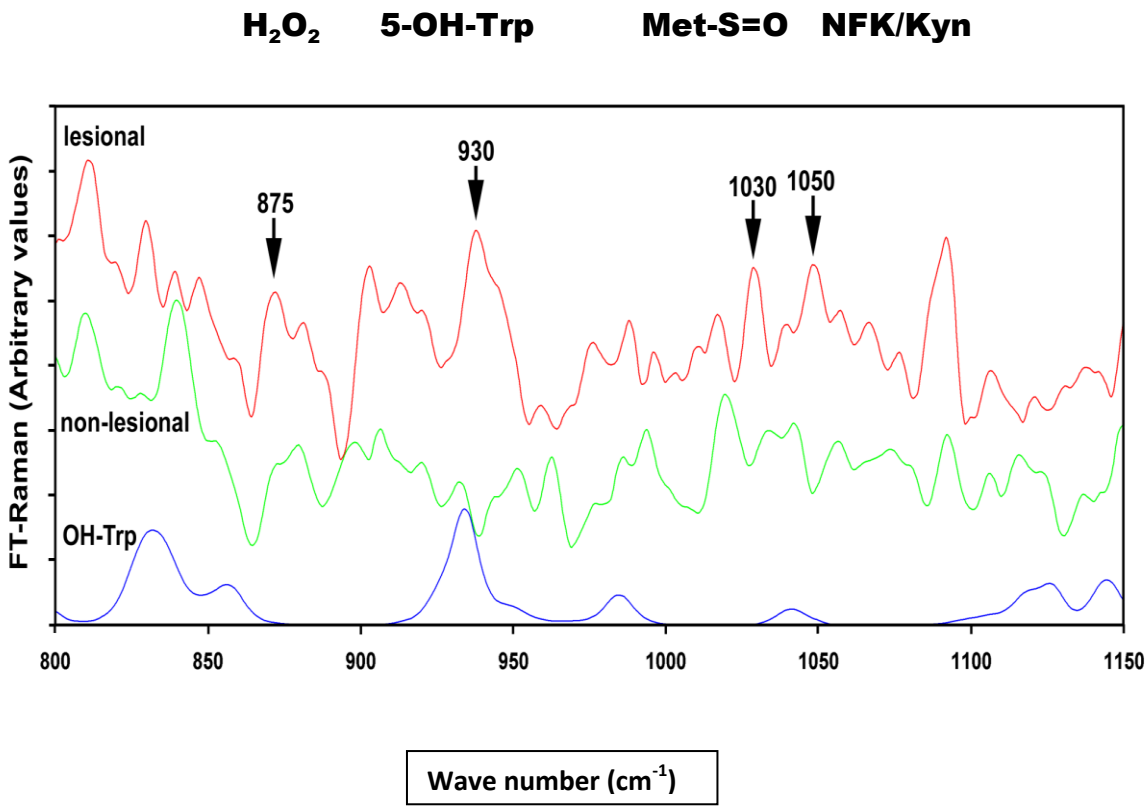

Figure 71: *In vivo* FT-Raman spectrum in MAL.

The spectra, obtained from lesional and non-lesional skin of MAL, provide evidence for epidermal accumulation of H_2O_2 and its oxidation products including 5-OH-tryptophan (5-OH-Trp), methionine sulfoxide (Met-S=O) and N-formyl kynurenine / kynurenine (NFK/Kyn) at the assigned wave numbers cm^{-1} Schallreuter et al ., 2012a).

9.2 Evaluation of epidermal catalase, p53 and p21 in MAL

9.2.1 Catalase expression follows a gradient from the basal layer to the upper layers and is lower in lesional and non-lesional skin of MAL compared to control skin.

Nowadays it is a fact that acute vitiligo is characterised by the presence of high epidermal H_2O_2-levels, associated with significantly decreased catalase expression throughout the entire epidermal compartment in both, lesional and non-lesional skin (Schallreuter et al, 1991, Schallreuter et al., 2008, Salem et al 2009, Schallreuter, 2014). Consequently it was tempting to use this protein as a marker to follow catalase in MAL.

Our *in situ* results show a down-regulation of epidermal catalase in lesional (d) and non-lesional skin of MAL compared to control skin (a). There is one fundamental difference. Catalase expression in MAL is much higher in the basal/suprabasal layer compared to the upper layers (**Figure 72)**.

Image analysis of the protein expression confirms a significant decrease in lesional and non-lesional skin compared to healthy controls with significantly higher expression in basal/suprabasal layers than in the upper layers in MAL (NS $p>0.05$, ** $p>0.01$, ***$p<0.001$, mean ± SE) **(Figure 73)**. The results are based on 3 intra- individual repeats of immuno-fluorescence labelling.

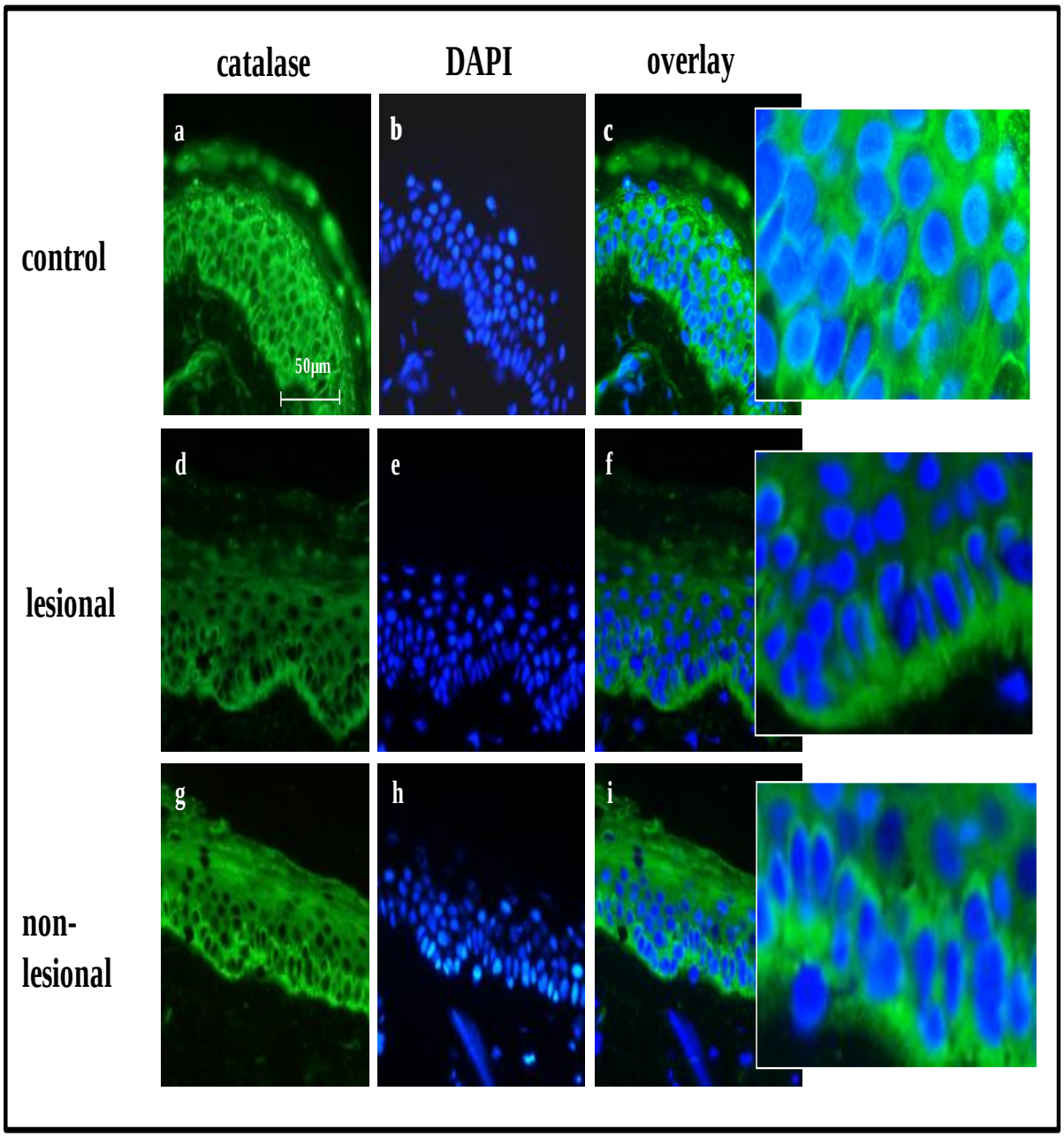

Figure 72: *In situ* expression of catalase in MAL.

Immuno-reactivity staining (FITC-labelling, green) shows lower protein expression in lesional (d) and non-lesional epidermis (g) compared to control skin (a). Expression follows a gradient. Catalase expression is more pronounced in the basal / suprabasal layer of non-lesional (g) and lesional skin (d). N.B. Controls (a) do not show this gradient. Scale bar 50µm. Magnification x 400.

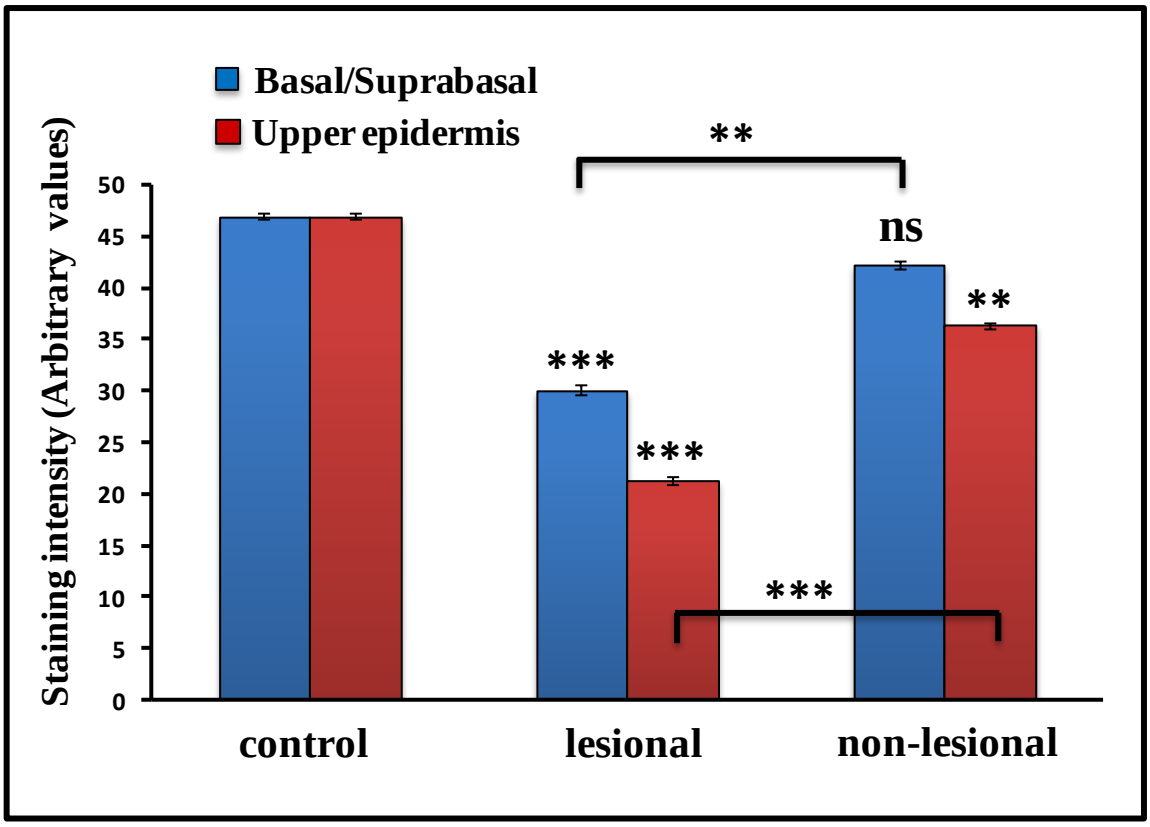

Figure 73: Image analysis of catalase protein levels in MAL.

The average fluorescence intensity reveals significant down-regulation of catalase levels in lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) epidermis of the patient with MAL compared to controls (n=24: 4 individuals, 6 pictures each) There is a significant increase in the basal layer of lesional and non-lesional skin. Plots are mean ± SE (NS $p>0.05$, ** $p<0.01$, ***$p<0.001$).

9.2.2 Catalase expression is absent in epidermal melanocytes of MAL

In situ double immuno-fluorescence labelling with the melanocyte specific marker NKI / beteb1 and catalase, shows no catalase expression in epidermal melanocytes of non-lesional skin (**Figure 74),** while the protein is highly expressed in melanocytes of healthy controls.

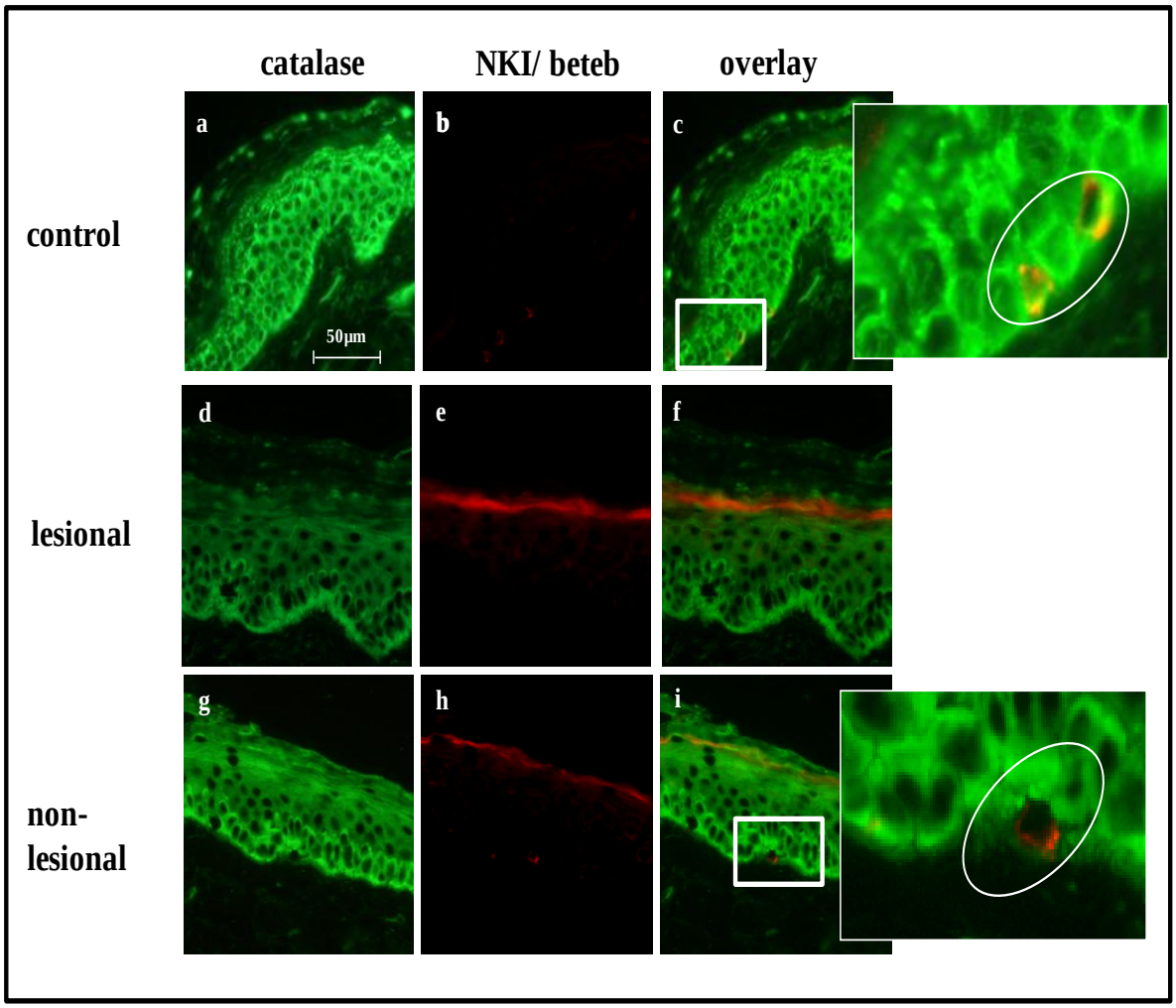

Figure 74: Absent catalase expression in epidermal melanocytes of MAL.

Immuno-reactivity (FITC-labelling, green) shows expression of catalase in MAL and in control skin. Melanocytes are detected with TRITC-labelled NKI / beteb1. Overlay with catalase shows the presence of the enzyme (yellow) in control skin ((c) / insert), while catalase expression is absent in non-lesional skin ((i)/insert). Scale bar 50µm. Magnification x 400.

Taken together, MAL shows significantly lower epidermal catalase expression with a distinct gradient from the basal / suprabasal layers to the upper layers compared to control skin. Expression levels are significantly higher in MAL compared to classical vitiligo.

Epidermal melanocytes show no catalase expression in MAL. This finding is not different from classical vitiligo.

9.2.3 Evaluation of p53 and p21 expression in lesional and non-lesional skin of MAL

9.2.3.1 Lower basal/suprabasal p53 expression is accompanied with high basal / suprabasal p21 expression in MAL

It has been reported that wild type functioning p53 is highly expressed in melanoma, predominantly with nuclear localization (Yamamoto and Takahashi, 1993; Sparrow et al., 1995; Zerp et al., 1999; Gwosdz et al., 2006). Expression appears to be increased with tumour progression and invasion. Hence, high p53 expression is considered to be a poor prognostic sign (Yamamoto and Takahashi, 1993; Montano et al., 1994; Sparrow et al., 1995; Zerp et al., 1999; Rass et al., 2001; Soussi and Beroud, 2001; Soto et al., 2005; Gwosdz et al., 2006; Li et al., 2006). Avery-Kiejda and colleagues reported in 2011, that despite high p53 expression, target genes, including p21, were severely altered. p53 inhibition in melanoma cell lines had no significant effect on its target genes. Therefore, it was suggested that p53 in melanoma may have lost its function to regulate target genes and rather than acting as a tumour suppressor, it may promote melanoma proliferation and progression (Avery-Kiejda et al., 2011).

Our own *in situ* data show lower p53 expression in basal / suprabasal layers than in the upper epidermal layers in both, lesional (d) and non-lesional skin (g) compared to control skin (a) **(Figure 75)**. Image analysis of epidermal p53-expression proves a significantly up-regulated protein expression. However, expression is higher in the upper epidermis compared to the basal / suprabasal layers. There are no significant differences in p53 levels between lesional and non- lesional skin (NS $p>0.05$, mean ±SE) **(Figure 76).** The data are based on 3 intra -individual staining experiments of the same sample.

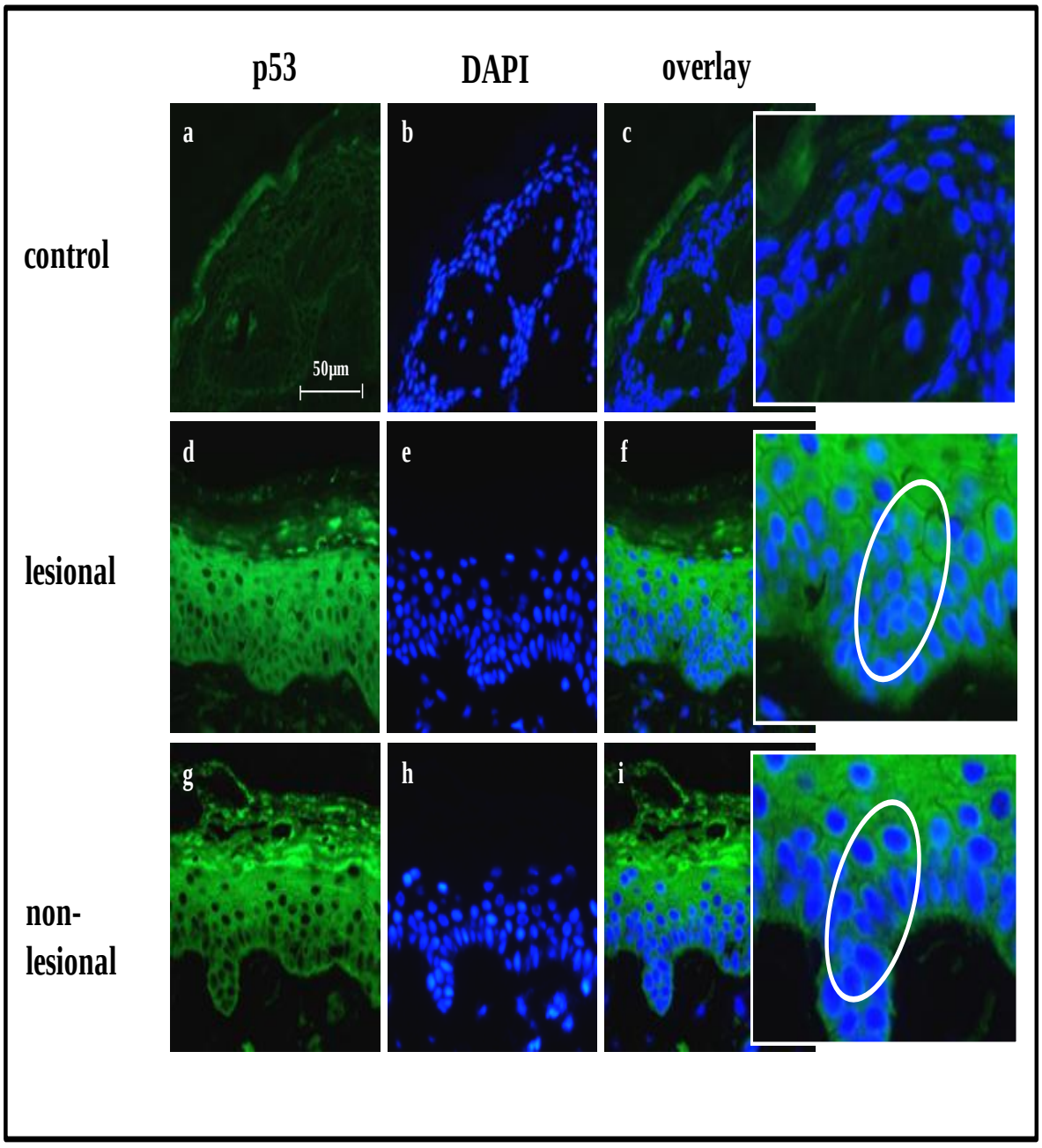

Figure 75: High expression of p53 in epidermis of MAL.

Immuno-reactivity (FITC-labelling, green) shows high expression of p53 in lesional (d) and non-lesional (g) skin of the patient compared to control skin (a). p53 expression appears lower in basal / suprabasal layers. p53 is predominantly expressed in the cytosol together with little nuclear expression (inserts f, i:, light blue after overlay with DAPI). Scale bar 50µm. Magnification x 400.

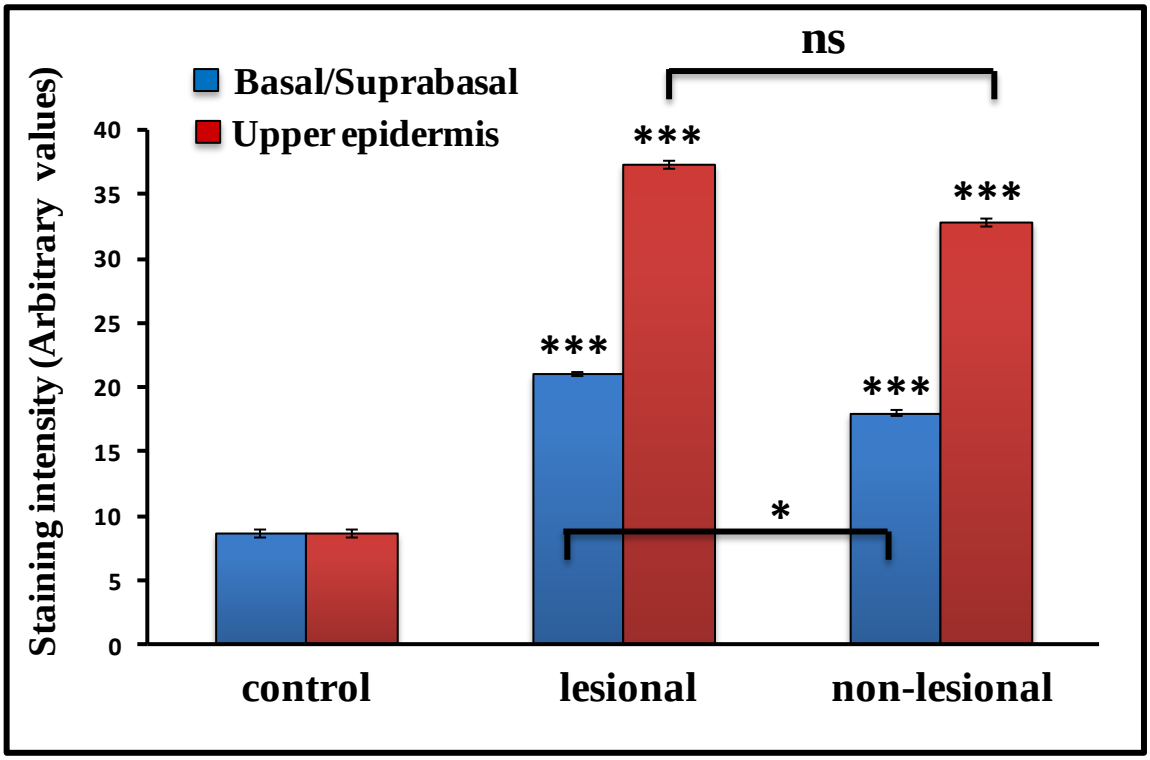

Figure 76: Significantly increased p53 expression in MAL.

Image analysis of the average fluorescence intensity shows significantly increased levels of p53 in lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non - lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin compared to controls (n=18: 3 individuals, 6 pictures each) Levels are lower in basal / suprabasal layers than in the upper layers of epidermis. There are no significant differences between lesional and non - lesional skin. (Plots are mean of 3 different intra individual stainings ± SE) (*** $p<0.001$, * $p<0.05$, NS $p>0.05$).

To sum up, p53 shows a reciprocal pattern to catalase expression. Based on our results we can conclude that epidermal p53 expression in MAL follows a gradient with lower expression in the basal / suprabasal layers, while expression levels are significantly up-regulated in the upper layers. These results differ from our findings in classical vitiligo, where p53 expression is high throughout the entire epidermal compartment.

9.2.3.2 Weak p53 *in situ* expression in epidermal melanocytes in MAL and healthy controls

Next we looked at *in situ* p53-expression of melanocytes using double immuno-fluorescence with FITC- labelled p53 and TRITC-labelled NKI / beteb1. The overlay showed weak p53-expression in melanocytes in MAL compared to control skin **(Figure 77).** This result is different to classical vitiligo where p53 is highly expressed.

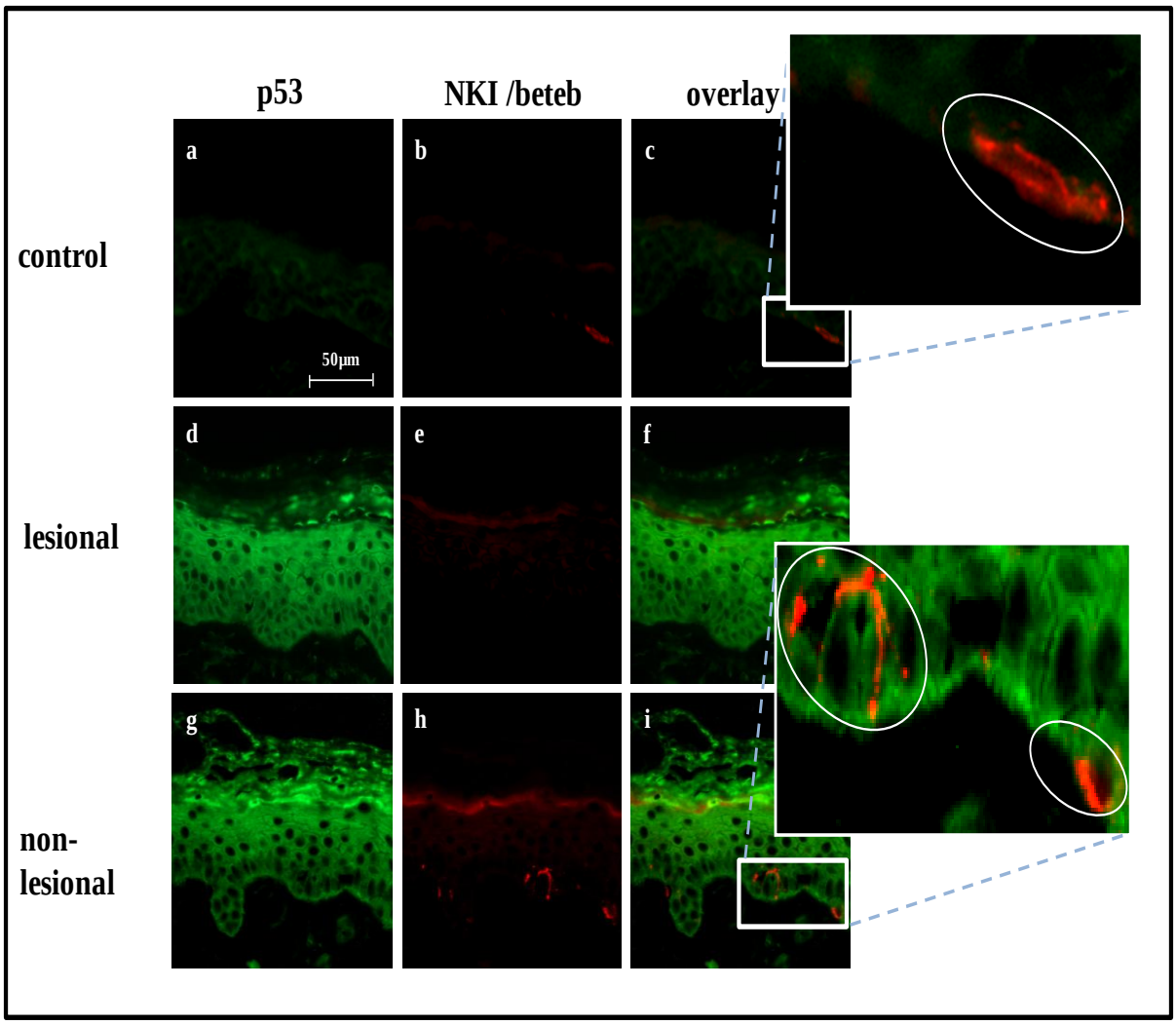

Figure 77: p53 in MAL is not expressed in melanocytes under *in situ* conditions. Immuno-reactivity (FITC- labelling, green) shows high p53 expression in lesional (d) and non- lesional (g) skin of the patient with MAL compared to the control (a). p53 is bearly expressed in NKI / beteb1/TRITC labelled melanocytes in MAL and controls (c, i, insert). Scale bar 50µm. Magnification x 400.

9.2.3.3 Western blot confirms significantly up-regulated p53 in MAL

In order to further substantiate the immuno-fluorescence data, we employed Western blot. The results confirm our observed up-regulated *in situ* p53 expression in both, lesional and non-lesional skin of MAL compared to control skin **(Figure 78a)**. Image analysis of p53 protein bands in relation to loading control protein (GAPDH) proves significantly increased p53 expression in MAL skin compared to skin of healthycontrols **(Figure 78b)**.

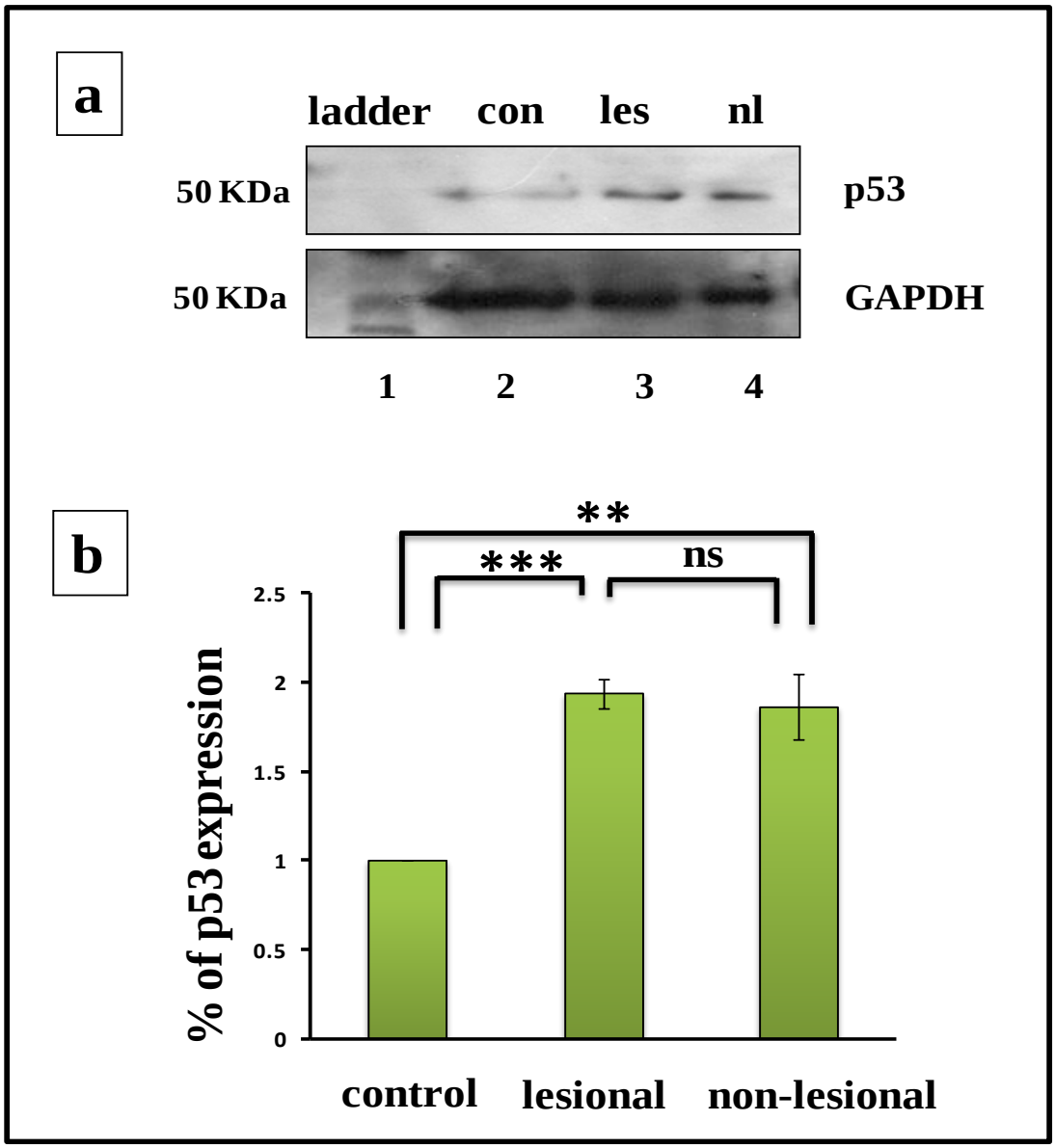

Figure 78: Significantly increased epidermal p53 protein expression in MAL.

(a) Western blot of p53 protein shows increased levels of its expression in both lesional and non-lesional skin of MAL compared to healthy control skin. Lane 1 protein ladder, lane 2 control skin, lanes 3 and 4 lesional and non- lesional skin tissue extracts from our patient with MAL. GAPDH was used as loading control.

(b) Quantification of p53 bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals significantly up-regulated expression in lesional and non- lesional skin of MAL compared to control skin. Plots are mean ± SE. (NS $p > 0.05$, ** $p < 0.01$, *** $p<0.001$).

9.2.3.4 Significantly increased epidermal p21 expression in basal / suprabasal layers of MAL

Next we tested *in situ* p21- expression in MAL. This protein is strongly expressed in the cytosol of the basal / suprabasal cells of lesional and non-lesional skin, while cells in upper layers reveal much weaker expression. Overlay with DAPI suggests some nuclear expression of p21 (**Figure 79).**

Image analysis of p21 confirms significantly up-regulated protein expression in basal / suprabasal layers of lesional and non-lesional skin (n=3) **(Figure 80)**. Analysis of the upper layers shows significantly higher expression in MAL compared to control skin **(Figure 80)**. Data are based on 3 intra-individual staining experiments of the same sample.

To sum up, the result in MAL is very different to classical vitiligo, where p21 is expressed throughout the entire epidermis without any significant differences between basal and suprabasal layers in lesional and non-lesional skin. The data in MAL suggest some nuclear expression of p21. Moreover, p21 expression in MAL does not follow the corresponding p53-expression. In fact, p21 shows a reciprocal expression pattern compared to p53 with high expression in basal / suprabasal layers and low expression in the upper epidermis.

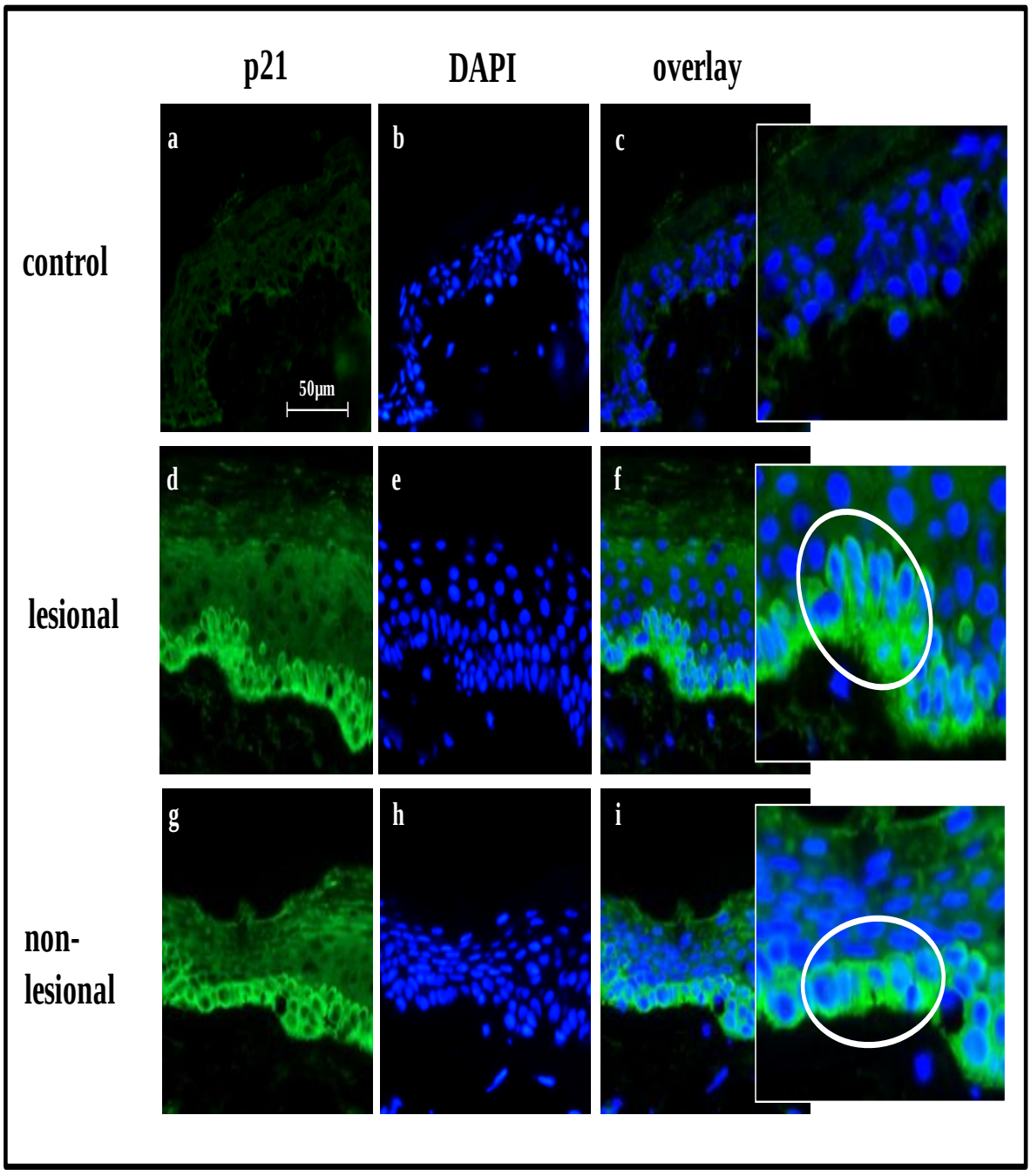

Figure 79: Up-regulated basal / suprabasal p21 expression in MAL.

Immuno-reactivity (FITC-labelling, green) shows p21 expression throughout the entire epidermis with stronger expression in the basal / suprabasal layer in lesional (d) and non-lesional (g) skin of MAL. p21 expression is absent in control skin (a).The overlay with DAPI suggests p21 expression in the nuclei of the basal layers (insert f, i light blue). Scale bar 50µm. Magnification x 400.

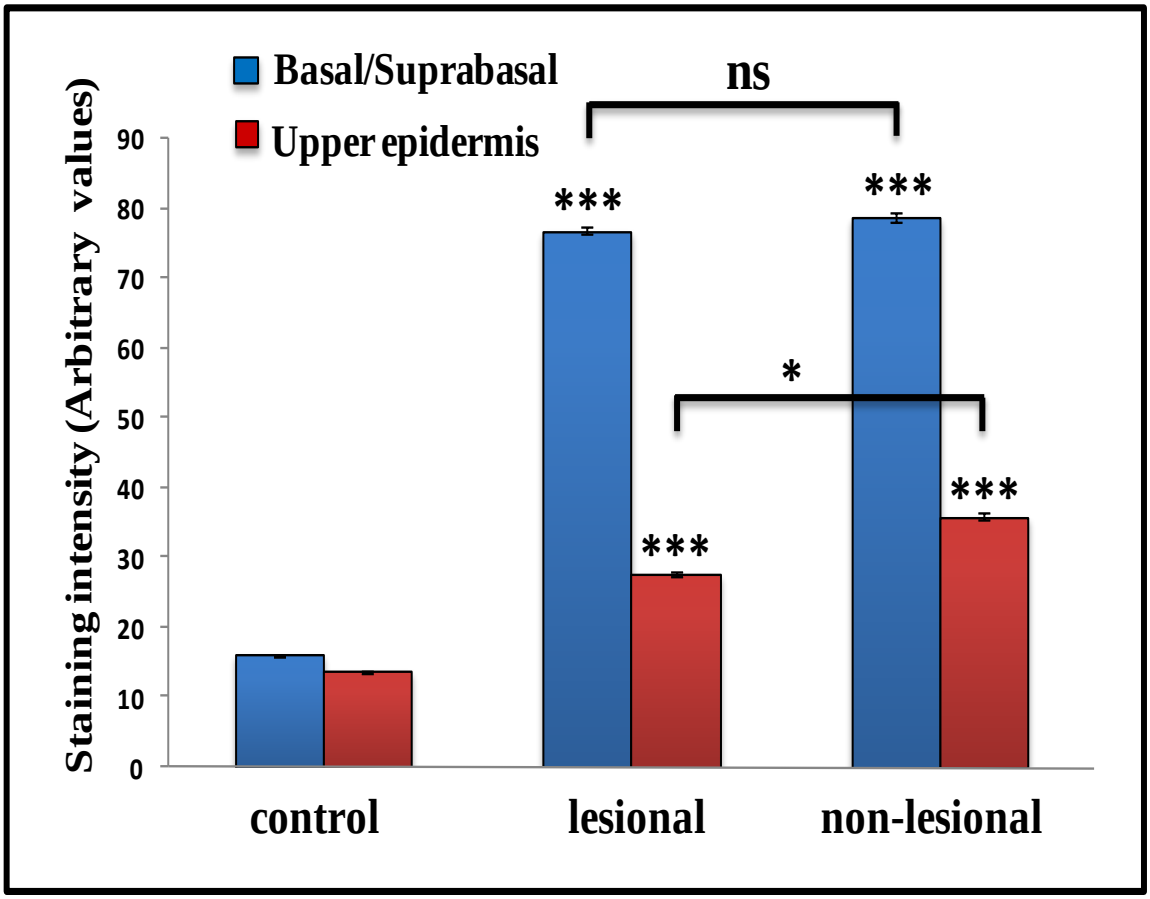

Figure 80: Significantly higher p21 expression in MAL.

Image analysis of the average fluorescence intensity reveals significantly increased p21 levels in basal / suprabasal layers of lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin in MAL compared to control (n=24: 3 individuals, 8 pictures each). Differences in p21 expression are not significant between lesional and non-lesional skin (blue columns). p21 is also expressed in the upper epidermis, although much weaker compared to the basal / suprabasal layers. The difference is still significant between patient and control skin (red columns). Plots are the mean ± SE (*** $p<0.001$, *$p<0.05$, NS $p>0.05$).

9.2.3.5 Western blot analysis confirms significantly increased epidermal p21 protein expression in MAL

In order to further substantiate the immuno-fluorescence data, we used again Western blot. The results confirm increased p21 expression in lesional and non-lesional skin of MAL compared to control skin **(Figure 81a)**. Image analysis of p21 protein bands in relation to loading control protein (GAPDH) confirms significantly increased p21 expression in lesional and non-lesional skin of MAL **(Figure 81b)**.

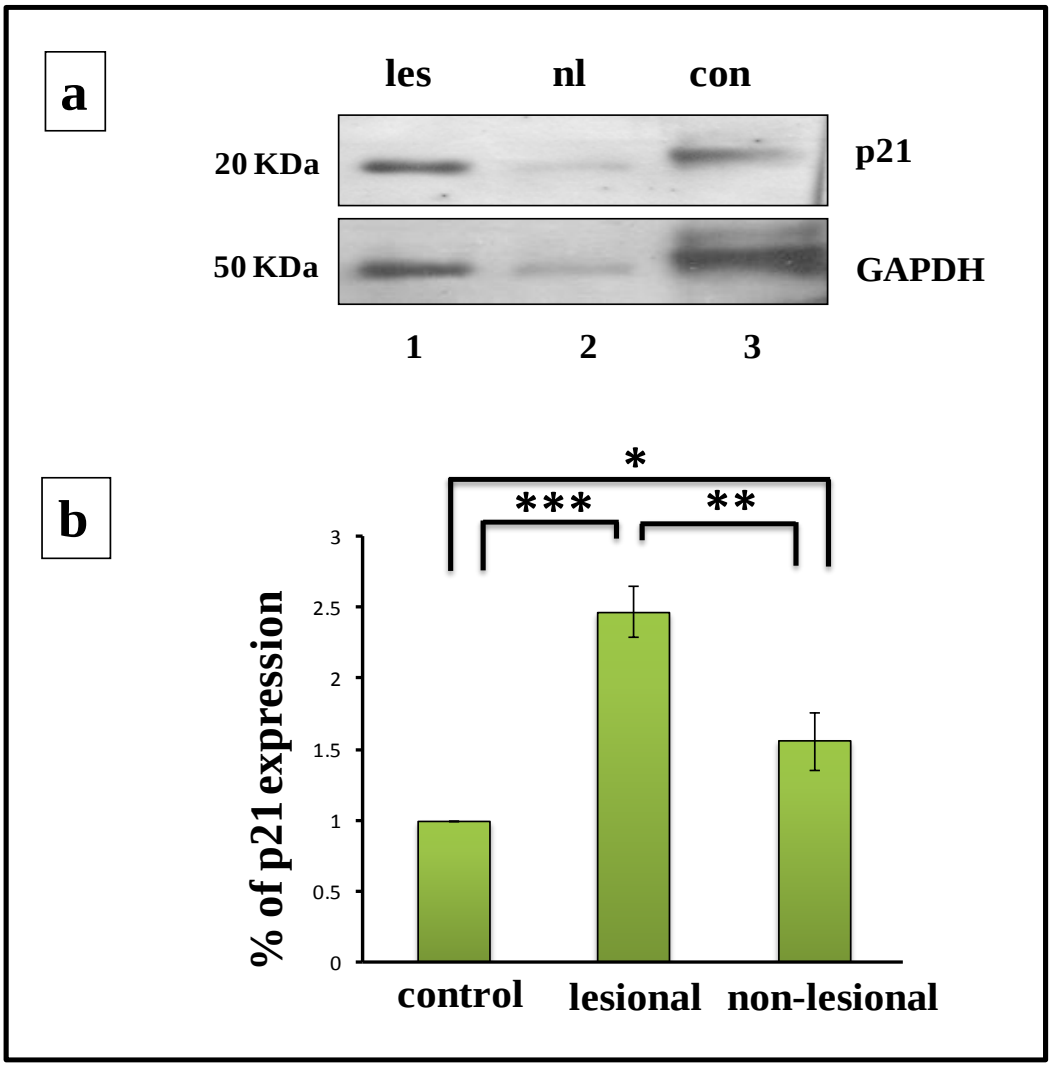

Figure 81: Significantly up-regulated p21 expression in skin of MAL.

(a) Western blot of p21 shows expression of p21protein in lesional and non-lesional skin of MAL compared to control skin. Lane 1 and 2 are lesional and non-lesional skin tissue extracts from patient with MAL (respectively), lane 3 control. GAPDH was used as loading control.

(b) Quantification of p21 bands. Image analysis was performed in relation to loading control protein GAPDH. The result reveals significantly increased protein expression in both, lesional and non- lesional skin of MAL compared to control skin (n=2). Plots are mean ± SE. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

9.2.3.6 *In situ* p21 expression in epidermal melanocytes of MAL and control skin

Next, we looked at possible p21 expression in epidermal melanocytes in our patient with MAL and in controls. p21 is weakly expressed in melanocytes of MAL non-lesional skin, but absent in controls as well as in classic vitiligo **(Figure 82).**

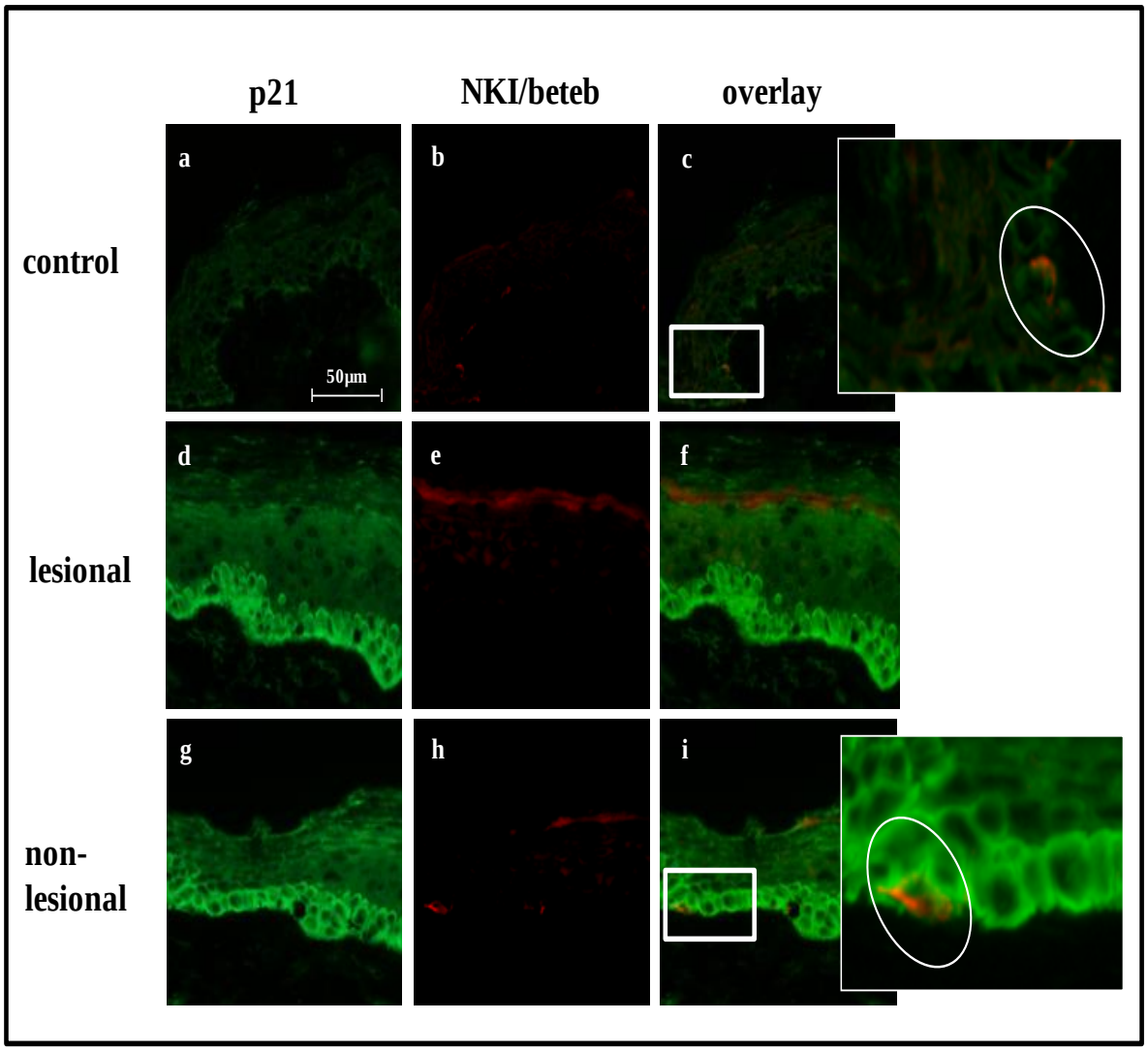

Figure 82: p21 expression in epidermal melanocytes of MAL and control skin.

Double immuno-reactivity staining (FITC- labelling, green) in the presence of TRITC / NKI / beteb1shows almost absent expression of p21 in melanocytes of MAL. p21 is completely absent in melanocytes of control skin after overlay of both chromophores (c, i and insert). Scale bar 50µm. Magnification x 400.

9.3 MDM2 and p53 regulation in MAL

Tumour suppressor gene (*TP53)* was found to be inactivated in many malignancies but rarely in malignant melanoma (Albino et al., 1994; Haluska et al., 1998). *MDM2* gene is amplified in more than 10% of 8000 human cancers from various sites, including e.g. lung and stomach (Toledo and Wahl, 2006). $p90^{MDM2}$ and its homologous MDM4 proteins may functionally co-operate or work separately in controlling p53 levels and activity (Honda et al., 1997; Fang et al., 2000; Pan et al., 2003). Furthermore, another member of MDM2 family, i.e. $p76^{MDM2}$, acts positively towards p53 via antagonizing the ability of $p90^{MDM2}$ to foster p53 protein degradation through inhibition of $p90^{MDM2}$ binding with p53, but without affecting $p90^{MDM2}$ levels (Perry et al., 2000). In 2001, Polsky and colleagues reported over-expressed MDM2 ($p90^{MDM2}$) protein in non-invasive melanoma, suggesting its involvement in early malignant transformation of melanocytes (Polsky et al., 2001). Moreover, expression of MDM2 ($p90^{MDM2}$) was found to be commonly amplified in invasive as well as metastatic melanoma (Polsky et al., 2001). In 2012, Gembarska and colleagues reported that MDM4 is a negative regulator of p53. Accordingly, it is over-expressed in about 65% of melanomas. Taking into consideration that TP53 mutations are rare in melanomas, these authors proposed that MDM4 over-expression is an important oncogenic event, altering p53 function in a large proportion of patients (Gembarska et al., 2012).

In order to get a better understanding of the reasons behind the reciprocal p53 and p21 expression in MAL as well as the possibility of p53 inactivation in the epidermis of MAL, we looked at p53-regulatory factors, including $p76^{MDM2}$, MDM4 and MDM4phospho.

9.3.1 High p76^{MDM2} expression in the basal / suprabasal layer of lesional MAL

Our *in situ* p76^{MDM2} immuno-fluorescence data demonstrated in vitiligo significantly higher levels of the p76^{MDM} MDM2 splice variant throughout the epidermis while this protein was absent in control skin. In MAL we see a higher expression in the basal / suprabasal layer compared to upper layers in lesional skin compared to controls. **(Figure 83)**. Image analysis of p76^{MDM2} expression proves significantly higher protein expression in lesional epidermis compared to control skin (* $p<0.05$, *** $p<0.001$, mean ±SE) (**Figure 84)**. Unfortunately, we have no data on non-lesional skin due to shortage of tissue.

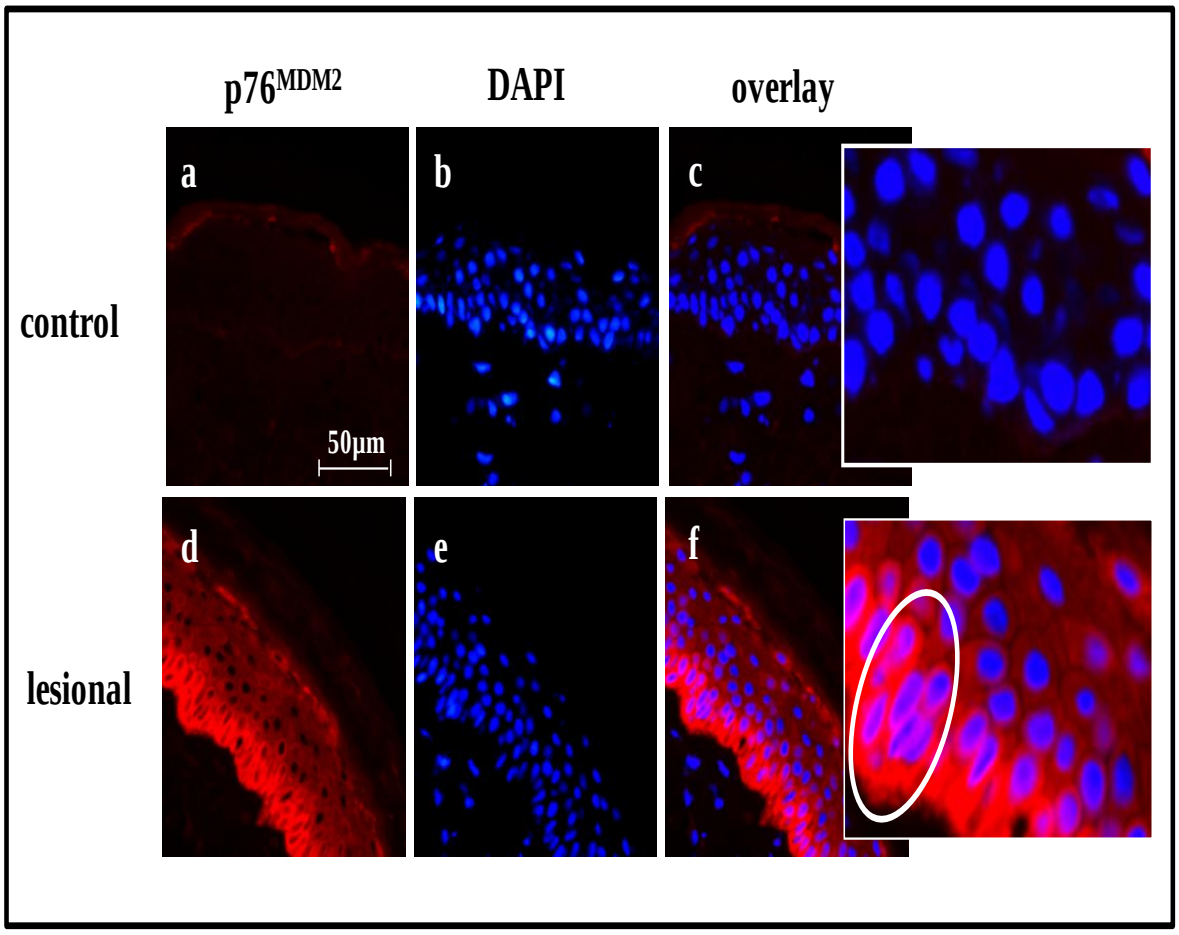

Figure 83: Up-regulated basal / suprabasal expression of p76^{MDM2} in lesional MAL.

Immuno-reactivity (TRITC-labelling, red) shows up-regulation in p76^{MDM2} expression of lesional (d) skin in MAL. This expression is absent in control skin (skin phototype III (a)). Expression of the protein is pronounced in the basal / suprabasal layers. Overlay of TRITC with DAPI suggests increased expression in the nuclei of epidermal cells with more extent in the basal and suprabasal cells identified by purple colour (f) and insert. Scale bar 50µm. Magnification x 400.

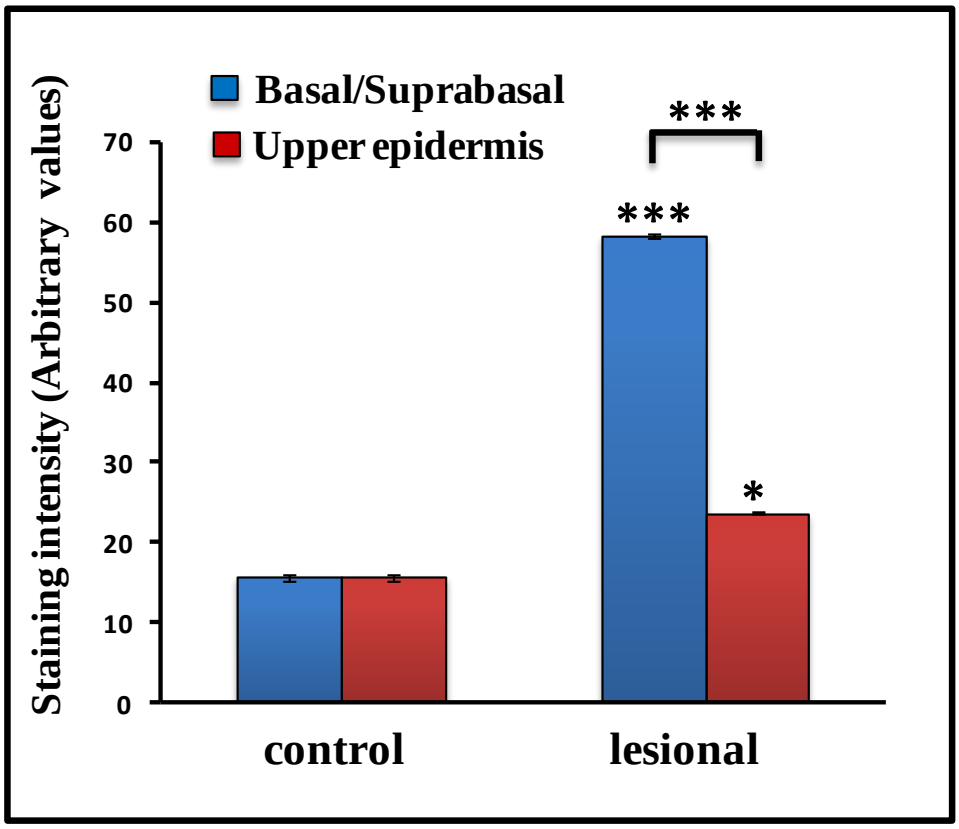

Figure 84: Significant increase in p76^{MDM2} expression in MAL.

Image analysis of the average fluorescence intensity shows significantly increased levels of p76^{MDM2} in epidermis of lesional skin of MAL (n=21: 3 intra-individual repeats, 7 pictures each) compared to healthy control skin (n=24: 3 individual repeats, 8 pictures each) This expression is significantly higher in basal / suprabasal layers than in the upper layers (Plots are mean ± SE) (* $p<0.05$, *** $p<0.001$).

9.3.2 Western blot analysis supports up-regulated p76^{MDM2}

The Western blot results show a significant increase in the expression of p76^{MDM2} in both lesional and non lesional skin of MAL, while it was almost undetectable in control skin **(Figure 85).** This result is not different from classical vitiligo.

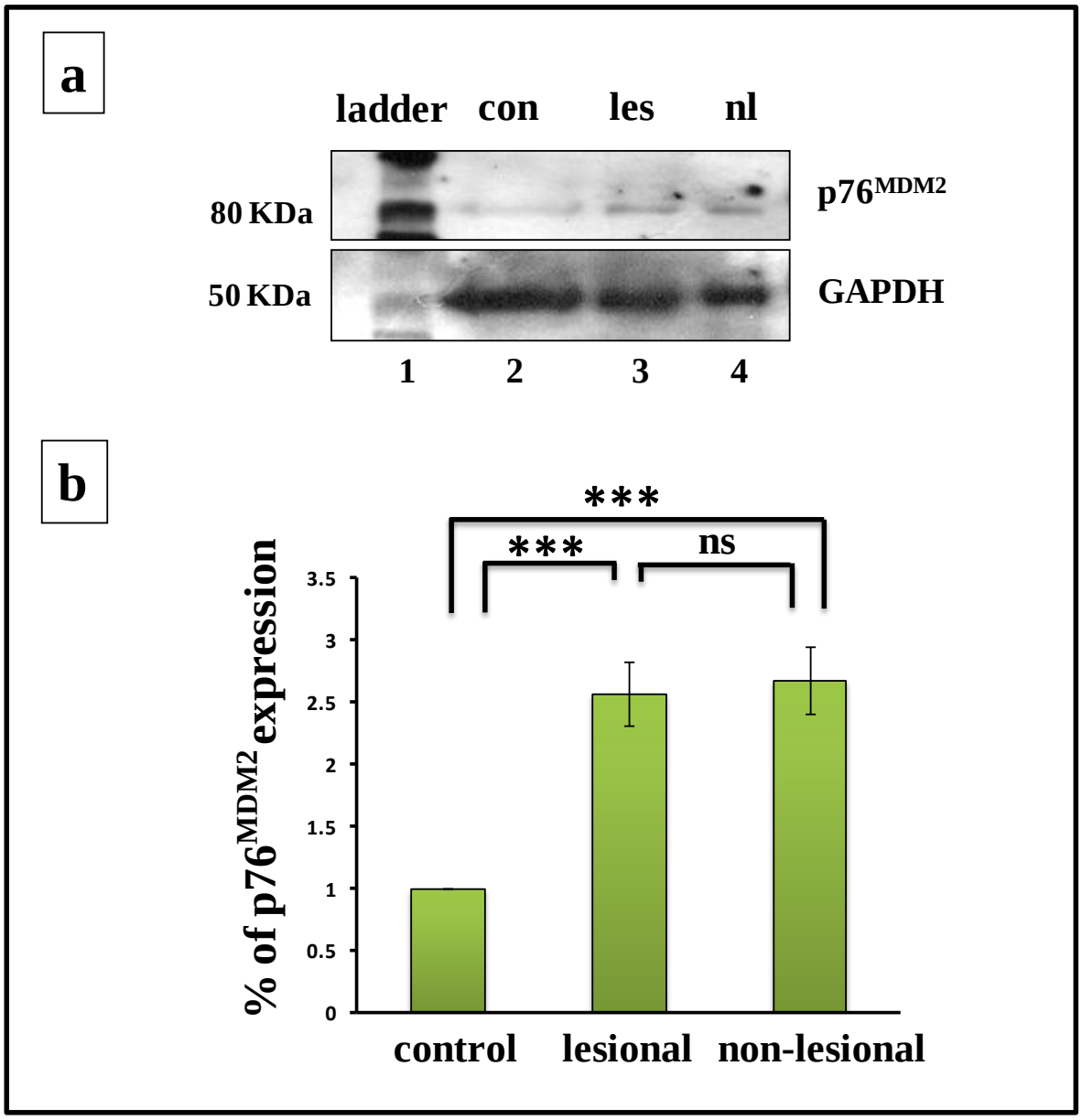

Figure 85: Up-regulated p76^{MDM2} expression in MAL.

(a)Western blot for MDM2 shows an elevation in p76^{MDM2} expression in lesional and non-lesional skin of MAL compared to control skin. Lane 1 protein ladder, Lane 2 control, Lanes 3-4 lesional and non-lesional skin extracts from MAL. GAPDH was used as loading control.

(**b) Quantification of p76^{MDM2} expression.** Image analysis of bands was used to quantify expression of the protein in lesional and non-lesional skin in relation to its expression in control skin. The result reveals significant up-regulated p76^{MDM2} expression in MAL compared to normal healthy control (NS > 0.05, *** p<0.001).

9.3.3 Significantly increased epidermal MDM4 expression in MAL shows a gradient from the basal / suprabasal layer to the upper layers

Next, we examined a possible role for MDM4 in controlling p53 in MAL. To do so, MDM4 and its phosphorylated protein MDM4phospho were studied in lesional and non-lesional skin of our patient with MAL and the results were compared to control skin. Our *in situ* MDM4 immuno-fluorescence staining results show increased MDM4 expression in both, lesional and non-lesional skin. MDM4 is also expressed in the nuclei of basal and suprabasal cells of MAL skin with higher extent in lesional skin **(Figure 86).** Image analysis of MDM4 proves significantly up-regulated expression of MDM4 in the entire epidermis of lesional and non-lesional skin (NS >0.05, ** $p<0.01$, *** $p<0.001$, mean ±SE) (**Figure 87)**.

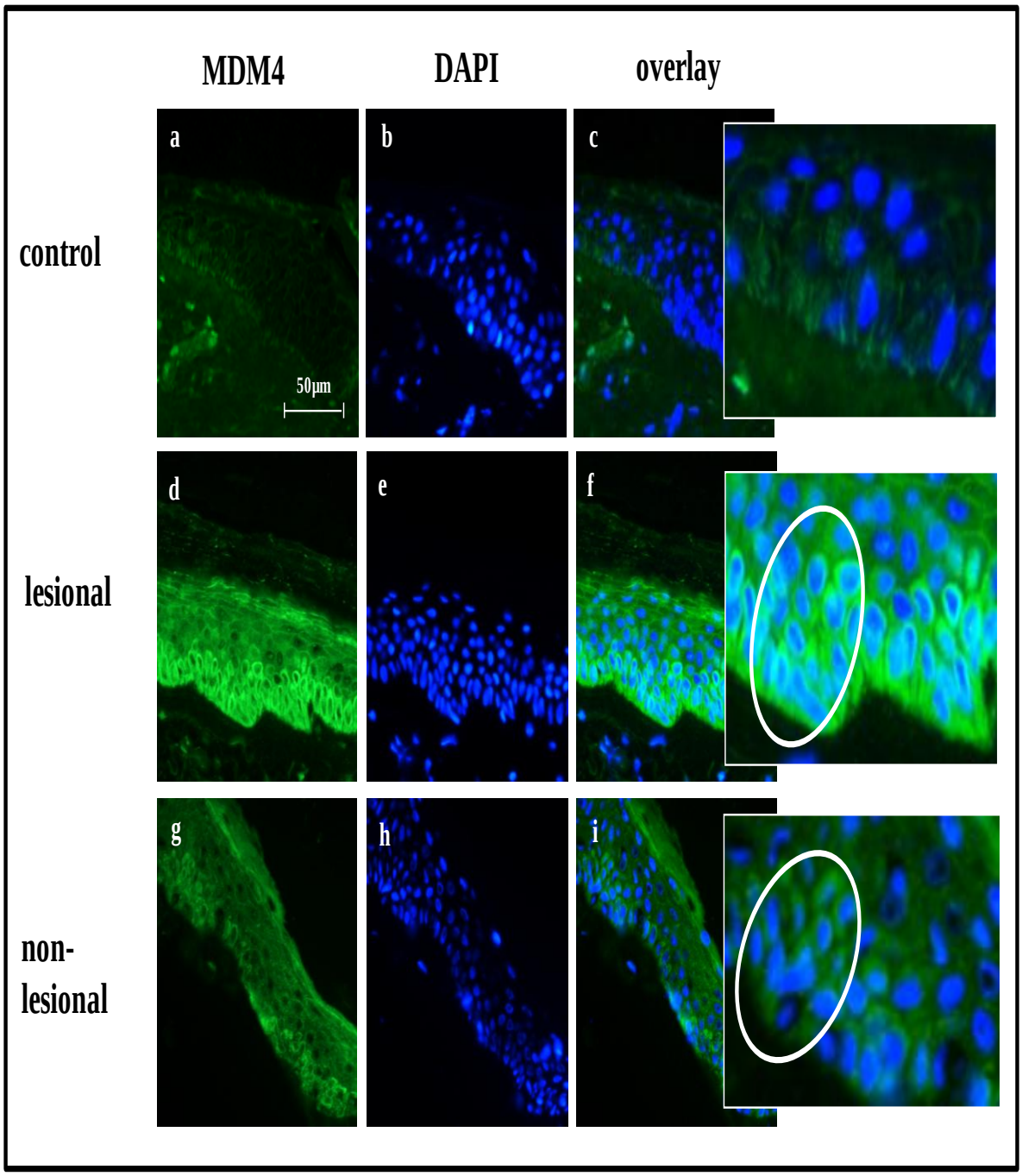

Figure 86: Increased expression of MDM4 in MAL.

Immuno-reactivity staining (FITC- labelling, green) shows considerable increase in the expression of MDM4 in the epidermis of both lesional (d) and non-lesional (g) skin of MAL compared to control with skin phototype III (a).N.B. This expression is more pronounced in the basal / suprabasal layers. Scale bar 50µm. Magnification x 400.

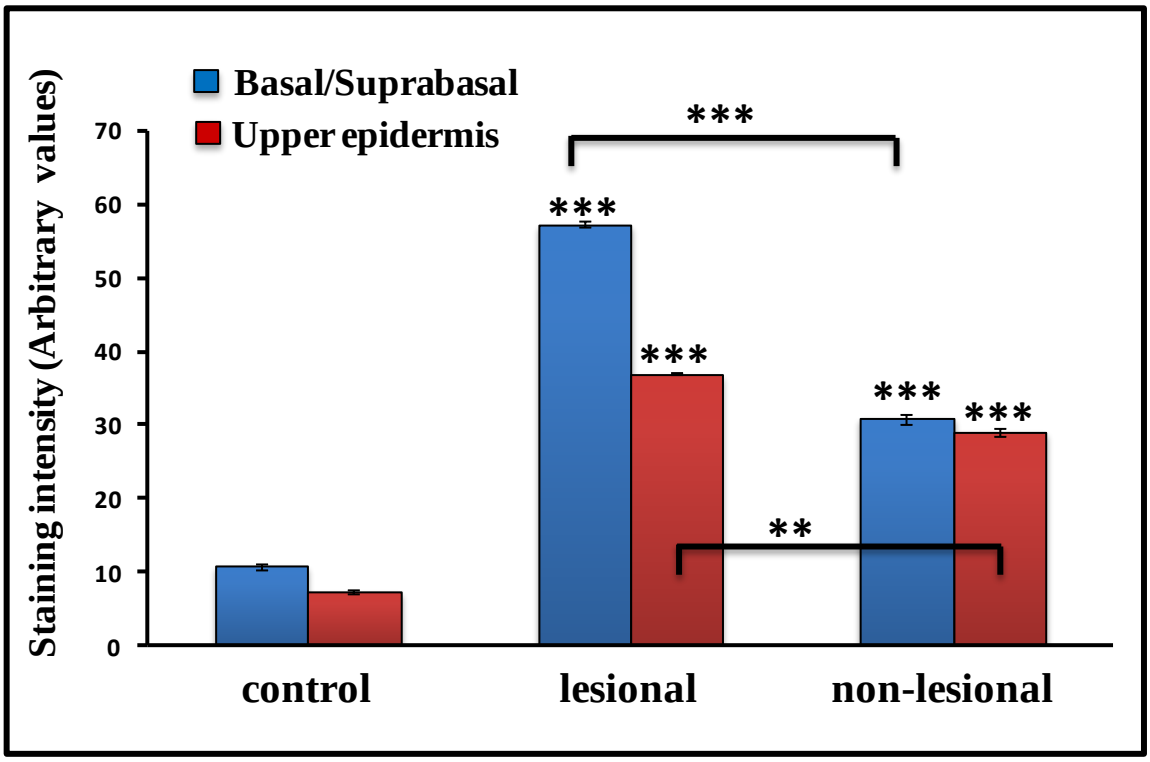

Figure 87: Significant increase of MDM4 expression in MAL.

Image analysis of the average fluorescence intensity reveals significantly increased MDM4 levels in lesional (n=18: 3 intra-individual repeats, 6 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin of MAL compared to controls (n=24: 3 -individuals, 8 pictures each). Plots are mean ± SE) (*** $p<0.001$, ** $p<0.01$).

9.3.4 Melanocytes of non-lesional skin present MDM4 expression

We looked at *in situ* MDM4 expression in epidermal melanocytes in our patient with MAL and in controls. The protein is indeed expressed in MAL as well as in classic vitiligo, but absent in controls **(Figure 88).**

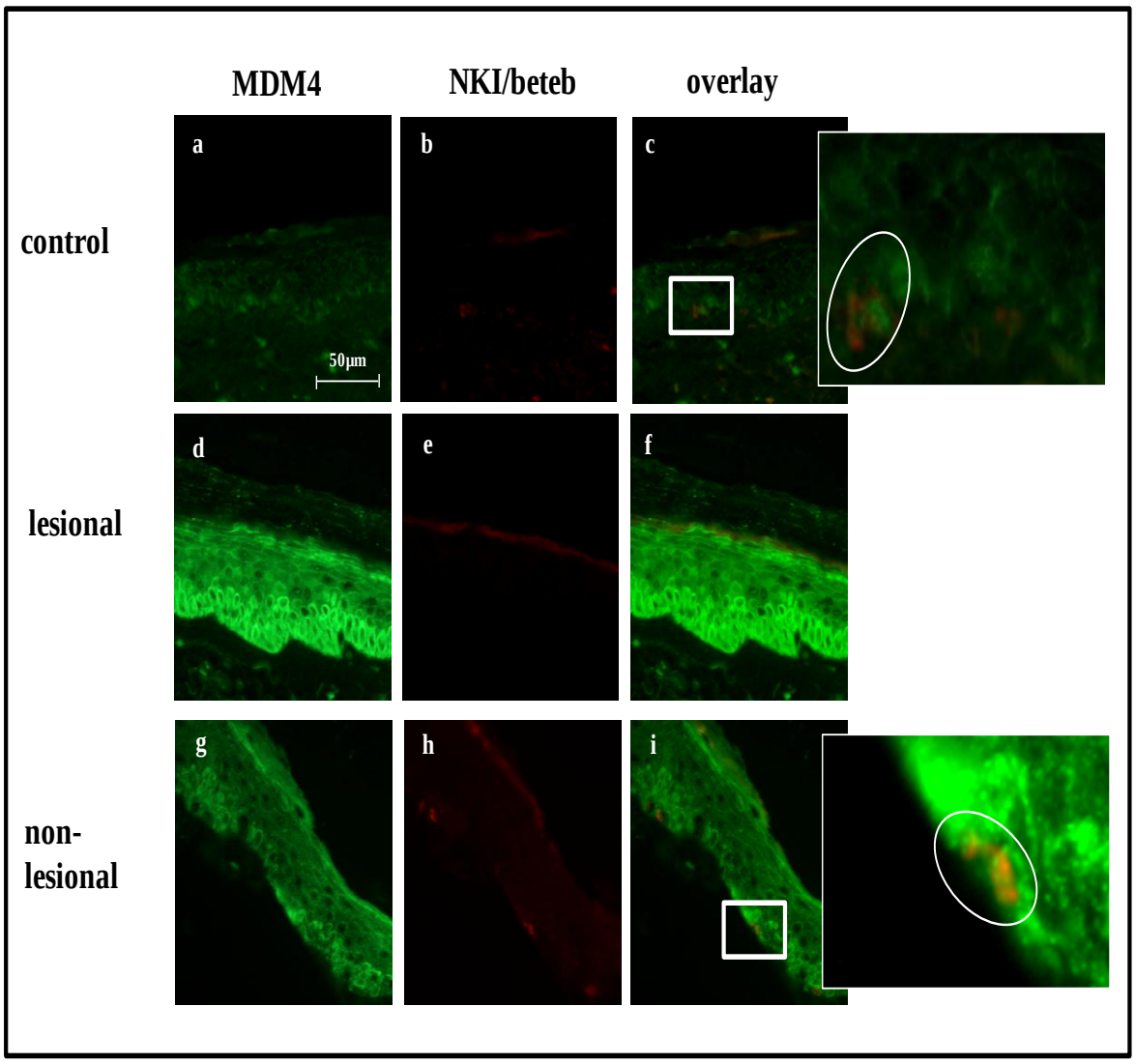

Figure 88: Evidence for MDM4 expression in melanocytes of MAL.

Immuno- reactivity staining (FITC-labelling, green) shows strong expression of MDM4 in the epidermis of both lesional (d) and non-lesional (g) skin of MAL compared to control skin (phototype III). Melanocytes of non-lesional MAL (i) show MDM4 expression, which is absent in normal controls (c). Scale bar 50µm. Magnification x 400.

9.3.5. Evidence for phosphorylation of MDM4 in MAL

MDM4 is a negative regulator of p53 (Gembarska et al., 2012). MDM4 looses this control based on phosphorylation in response to UVR (Jin et al., 2006). Taking these facts into consideration, it was tempting to evaluate the amount of phosphorylated MDM4 in MAL. Our results show very high phosphorylation levels of the protein throughout the entire epidermis **(Figures 89).** Image analysis of MDM4phospho proves significantly up-regulated expression of the protein in lesional and non-lesional skin compared to controls (* $p<0.05$, ** $p< 0.01$, *** $p<0.001$, mean ±SE) **(Figure 90)**. Calculation of the MDM4/MDM4phospho ratio reveals complete phosphorylation of the protein in the entire epidermis of MAL **(Table 9).** This result is different to classical vitiligo where MDM4 is not completely phosphorylated.

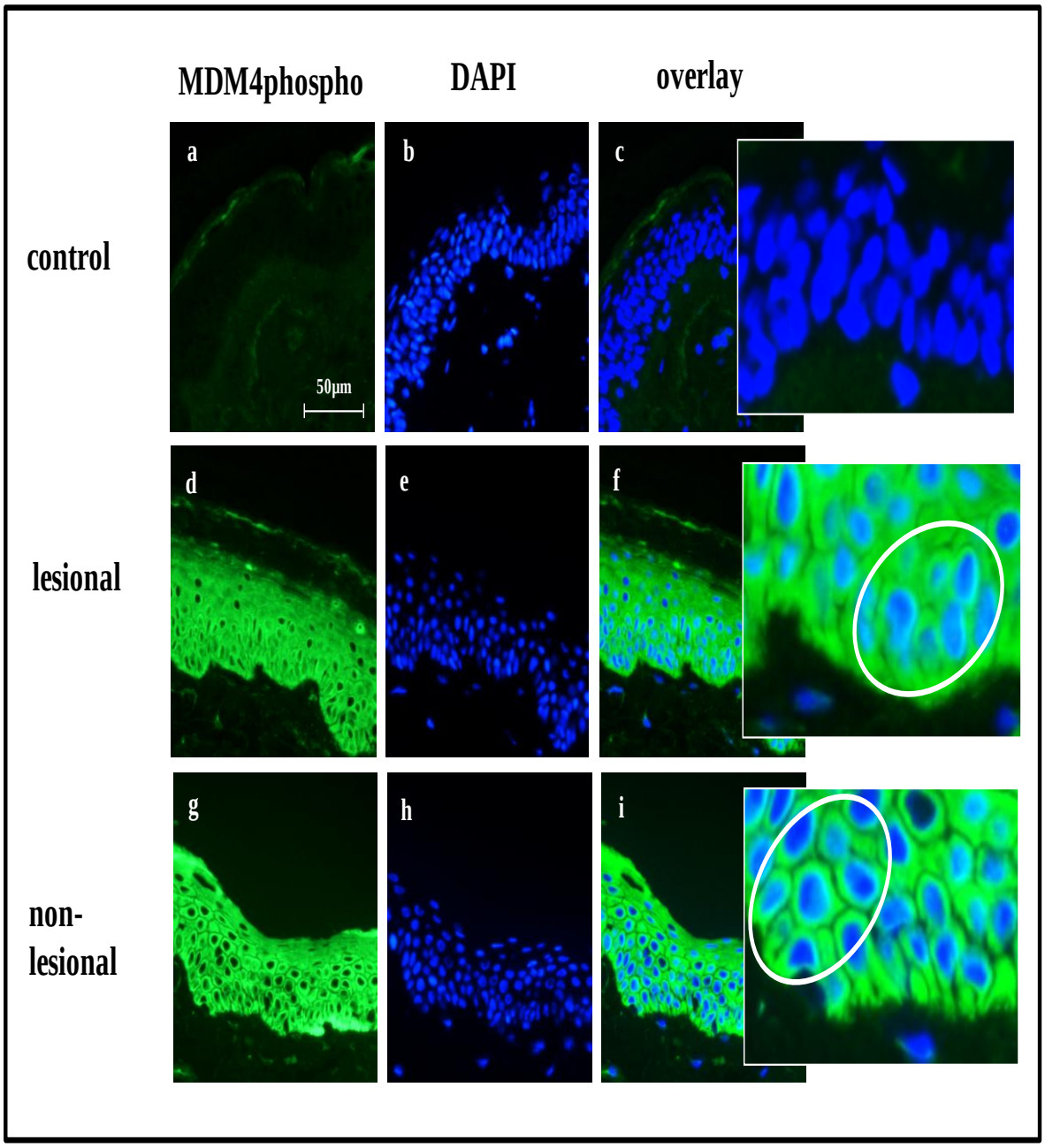

Figure 89: Up-regulated expression of MDM4phospho in MAL throughout the entire epidermis.

Immuno-reactivity staining (FITC-labelling, green) shows strong expression of MDM4phospho in the entire epidermal compartment of lesional (d) and non-lesional (g) skin in MAL compared to control skin (skin phototype III) (a). MDM4phospho seems to be present in nuclei with more extent in the depigmented skin (insert f, i). Scale bar 50µm. Magnification x 400.

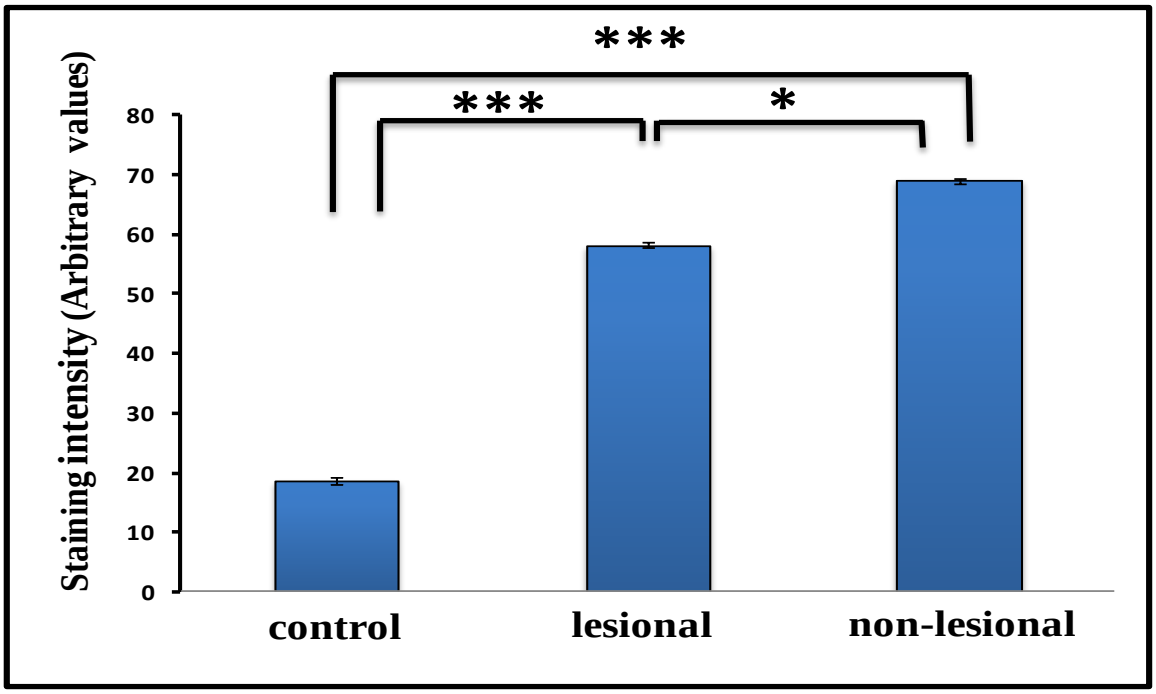

Figure 90: Significantly increased MDM4phospho expression in MAL.

Image analysis of the average fluorescence intensity shows significantly increased levels of MDM4phospho in lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin of MAL compared to controls (n=24: 3 individuals, 8 pictures each) (Plots are mean ± SE) (*** $p<0.001$, * $p<0.05$).

Protein	Epidermal layer	control	lesional	non-lesional
MDM4	basal	10.76	57.27	30.82
	upper	10.42	37.02	29.02
MDM4phospho	basal	18.7	68.99	58.24
	upper	18.7	68.99	58.25
MDM4phosph/MDM4	basal	1.74	1.20	1.89
	upper	1.79	1.86	2.01

Table 9

MDM4phospho-MDM4 immuno-fluorescence ratio in the basal / suprabasal and upper layers of MAL.

9.3.6 Melanocytes of non-lesional skin do not show MDM4phospho expression

Next we looked at *in situ* MDM4phospho expression in epidermal melanocytes in our patient with MAL and in controls. The protein is neither really expressed in MAL nor in control skin, but it is present in classical vitiligo **(Figure 91).**

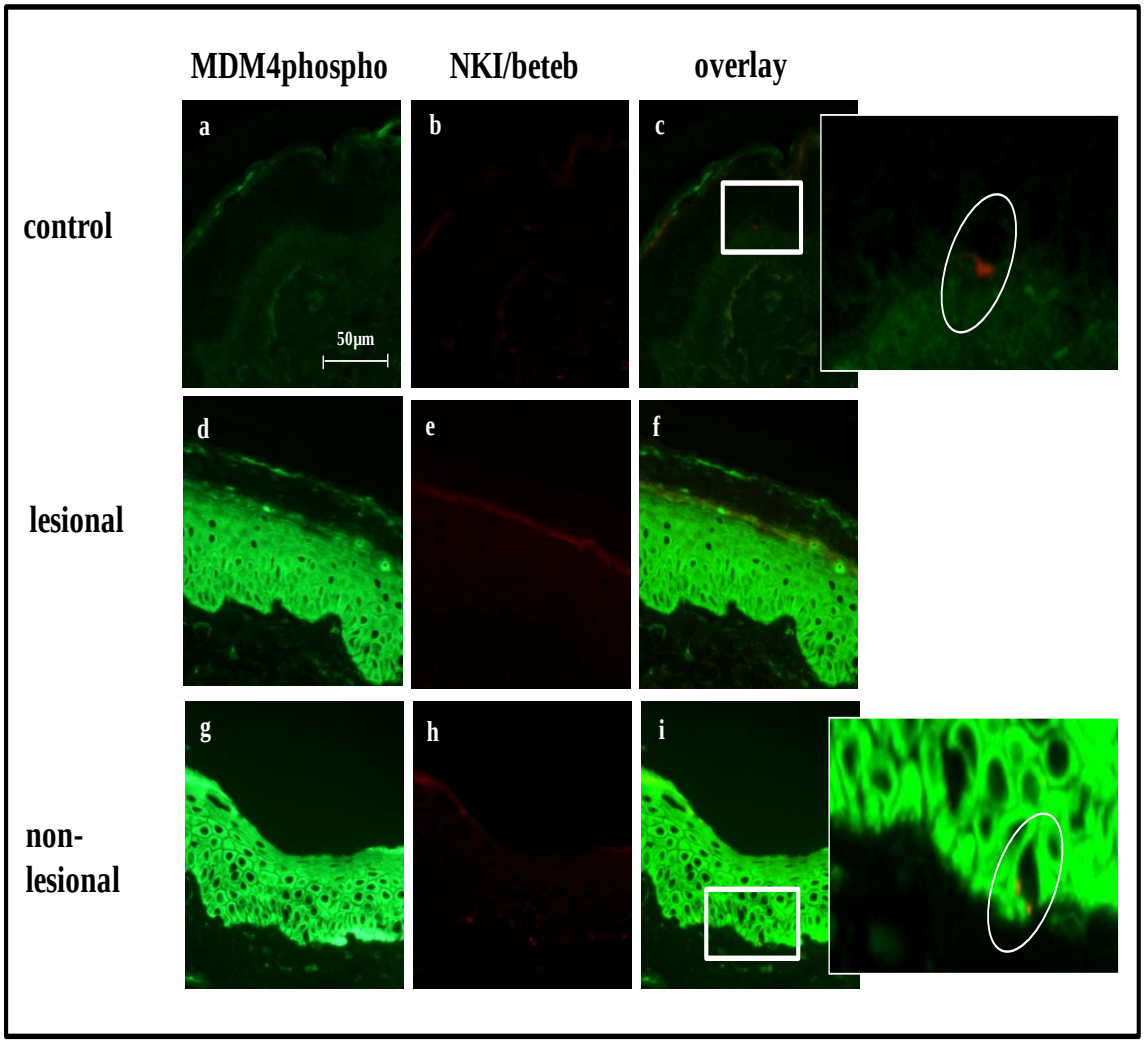

Figure 91: Weak expression of MDM4phospho in melanocytes of MAL.

Immuno-reactivity staining (FITC-labelling, green) shows high expression of MDM4phospho throughout the epidermis of both lesional (d) and non-lesional (g) skin of MAL compared to healthy control with skin phototype III. Melanocytes show very little MDM4phospho expression in non-lesional (i) and in control skin(c). Scale bar 50µm. Magnification x 400.

9.4 Expression of SPARC in lesional and non-lesional skin of MAL

9.4.1 Significant up-regulation in SPARC levels

To the best of our knowledge, the presence of SPARC in the epidermis of patients with classical vitiligo has been shown for the first time in the study presented herein **(Figures 43, 45)**. In 2011, the Fenouille group documented a direct relationship between SPARC and the p53 / p21 cascade. They recognised that SPARC inactivates p53 in melanoma, with subsequent inhibition of p21 production (Fenouille et al., 2011a;b). Based on these data together with our findings, it was tempting to imply a possible role for SPARC to explain the different p53 / p21 expression observed in MAL.

Our *in situ* results reveal high levels of SPARC throughout the entire lesional and non-lesional epidermis of our patient with MAL compared to control skin (**Figure 92).** Here it is of note that normal skin shows only background expression. Image analysis of SPARC confirms highly significant up-regulated protein expression in lesional and non-lesional skin compared to control skin (n=4) **(Figure 93)**.

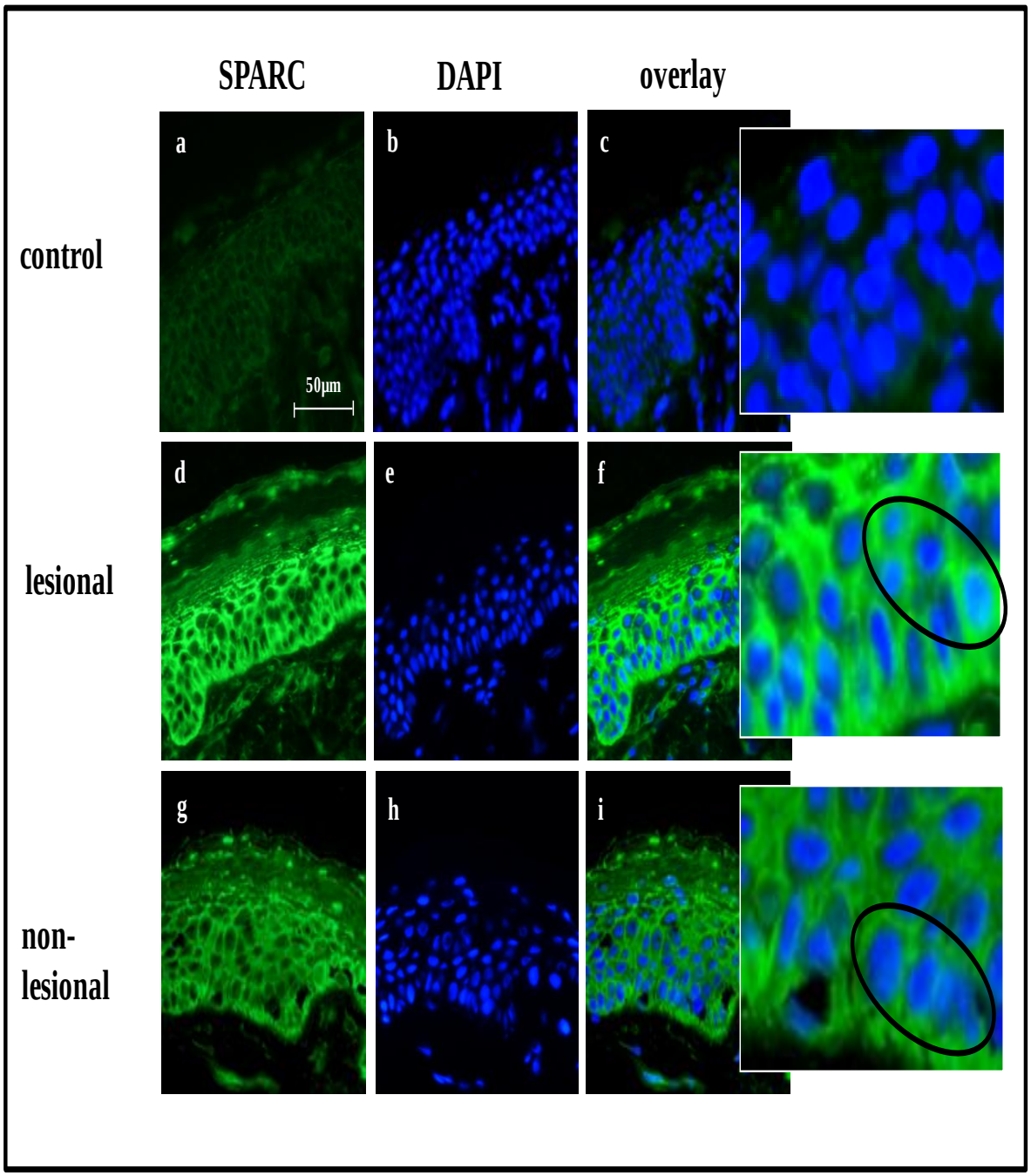

Figure 92: High expression of SPARC in MAL.

Immuno-reactivity staining (FITC-labelling, green) shows increased expression of SPARC in lesional (d) and non-lesional (g) skin of MAL compared to control skin (a). The nuclear expression seems to be stronger in lesional (insert f) compared to non-lesional skin. Scale bar 50µm. Magnification x400.

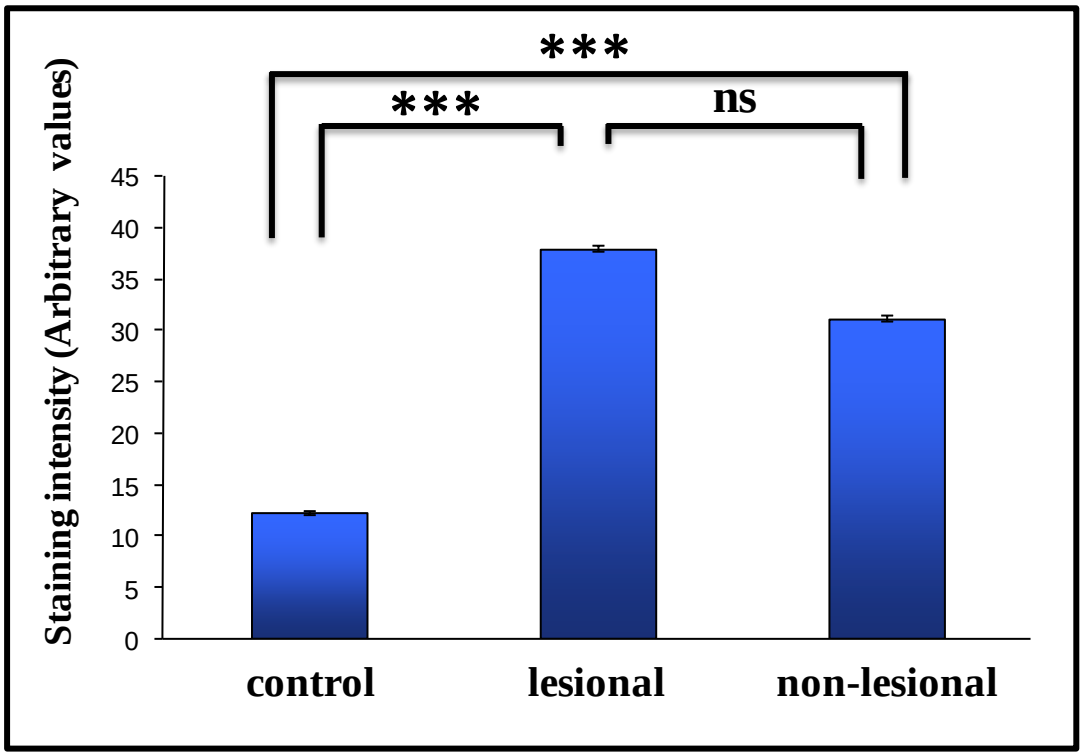

Figure 93: Significantly increased SPARC expression in MAL.

Image analysis of the average fluorescence intensity shows significantly increased levels of SPARC in lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin of MAL compared to controls (n=32: 4 intra-individual repeats, 8 pictures each). N.B. There are no significant differences between lesional and non-lesional skin. (Plots are mean ± SE) (*** $p<0.001$, NS $p>0.05$).

9.4.2 Western blot analysis confirms increased SPARC expression in MAL

In order to quantify protein expression, Western blot analysis was carried out. The results show increased SPARC expression in both lesional and non-lesional skin of MAL compared to skin of healthy control skin **(Figure 94a)**. Image analysis of SPARC protein bands in relation to loading control protein (GAPDH) reveals significantly up-regulated SPARC expression in the entire skin of MAL **(Figure 94b)**.

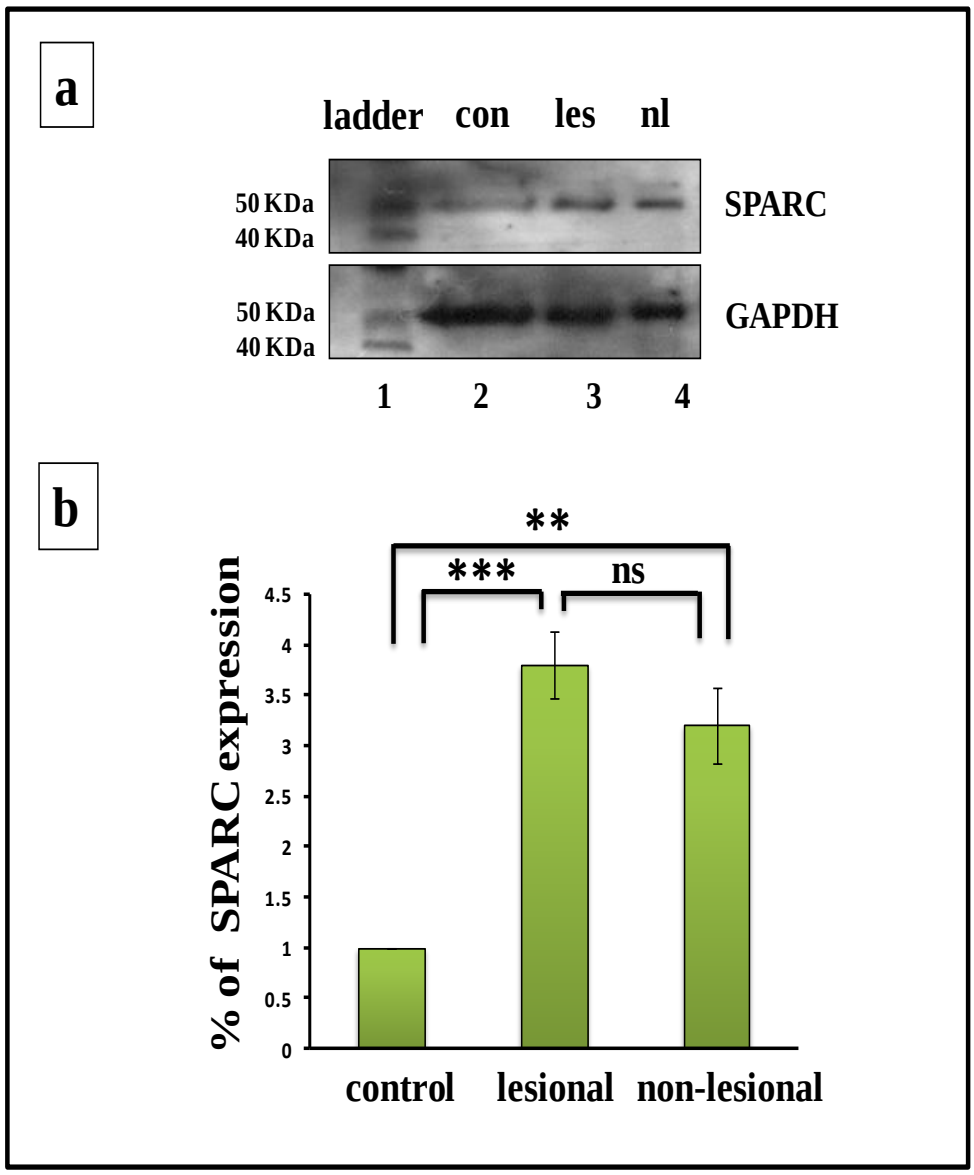

Figure 94: Significantly up-regulated SPARC levels in skin of MAL.

(a)Western blot. SPARC shows an increased expression in lesional and non-lesional skin of MAL compared to control skin. Lane1 protein ladder. Lane 2 control. lanes 3-4 lesional and non-lesional skin extracts from MAL. GAPDH was used as loading control.

(**b) Quantification of SPARC expression.** Image analysis of bands was used for quantification of protein expression in lesional and non-lesional skin in relation to GAPDH. The result reveals significantly up-regulated SPARC expression in lesional and non-lesional skin of MAL compared to normal control skin.

(NS $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$).

9.4.3 SPARC expression in epidermal melanocytes of MAL

Under *in situ* conditions melanocytes show the presence of SPARC expression in non-lesional skin of MAL (insert i), while SPARC is absent in melanocytes of control skin (insert c) (**Figure 95).**

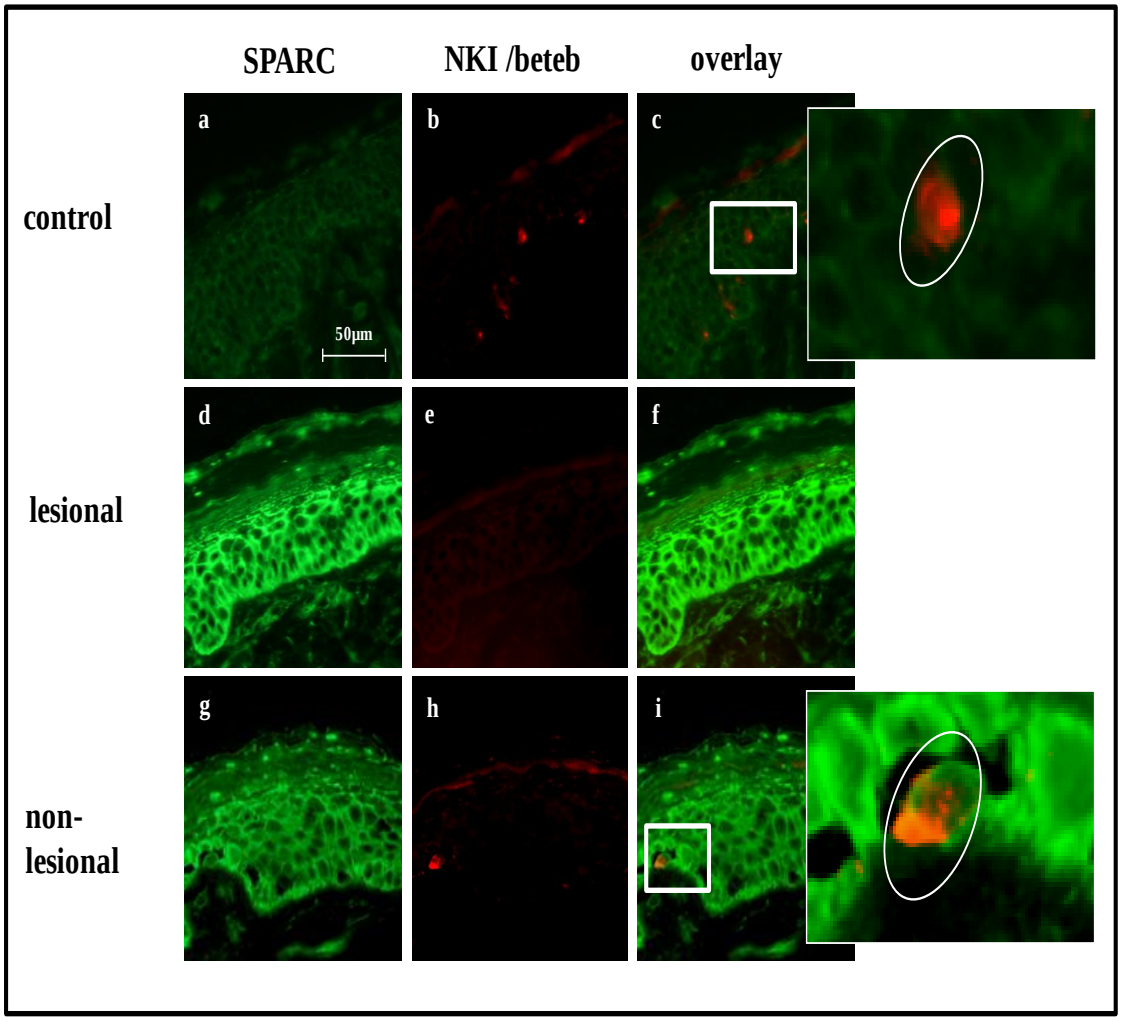

Figure 95: Weak *in situ* expression of SPARC in a dermal melanocyte of MAL.

Immuno-reactivity staining (FITC-labelling in green) shows high expression of SPARC throughout the entire epidermis in lesional (d) and non-lesional (g) skin of MAL compared to healthy control skin (a). One TRITC / NKI / beteb1 labelled detached dermal melanocyte of non-lesional skin reveals weak expression of SPARC (indicated by the orange and yellow fluorescence in the overlay) compared to normal controls (only red) (Scale bar 50µm). Magnification x400.

9.4.4 SPARC nitration in lesional and non-lesional skin of MAL

9.4.4.1 5-nitro-tyrosine expression in lesional and non-lesional skin

In classical vitiligo we showed the presence of high epidermal $ONOO^-$ levels in lesional and non-lesional skin (Salem et al., 2009 and in this thesis). Therefore it seemed intriguing to utilise 5-nitro tyrosine to look for possible epidermal $ONOO^-$ - expression in MAL.

Our *in situ* results reveal the presence of nitrated L-tyrosine expression in the skin of our MAL patient. This expression is weaker in basal and supra-basal layers than in the upper layers **(Figure 96).** Image analysis of the protein expression reveals significantly increased 5-nitro-tyrosine levels in lesional and non- lesional skin of MAL, compared to healthy controls (*** $p<0.001$, NS $p>0.05$, mean ± SE) **(Figure 97).**

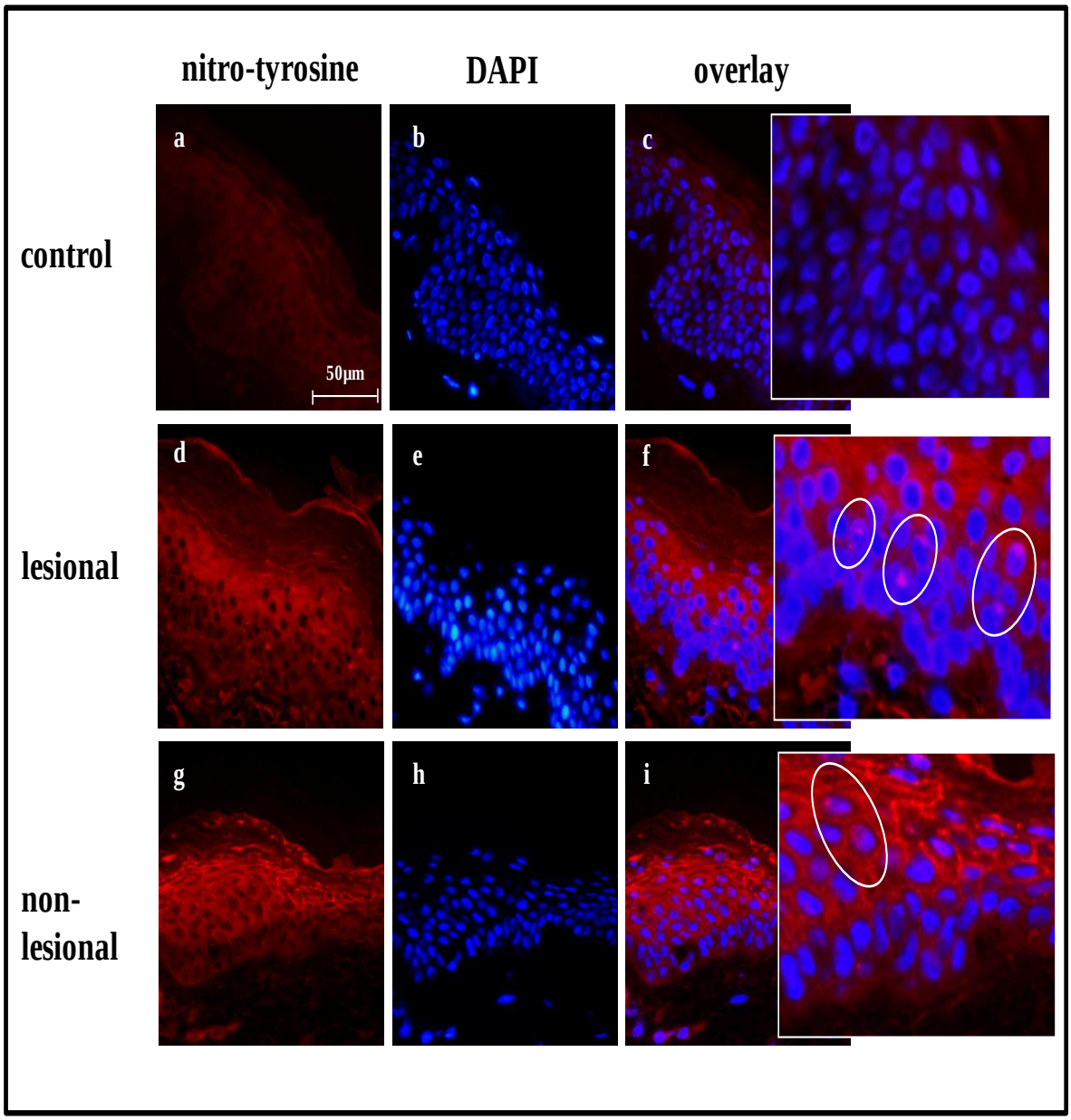

Figure 96: High 5- nitro-tyrosine expression indicates $ONOO^-$- formation in MAL.

Immuno-fluorescence reactivity of TRITC-labelled 5-nitro-tyrosine shows high expression of nitrated tyrosine in lesional (d) and non lesional (g) skin of MAL. Importantly, expression seems to follow a gradient with increased expression in the upper layers. Overlay of nitrated tyrosine with DAPI identifies its localization in some nuclei of non-lesional skin (purple) (i) compared to contro skin (c) (skin phototype III, Fitzpatrick classification). Scale bar 50µm. Magnification x 400.

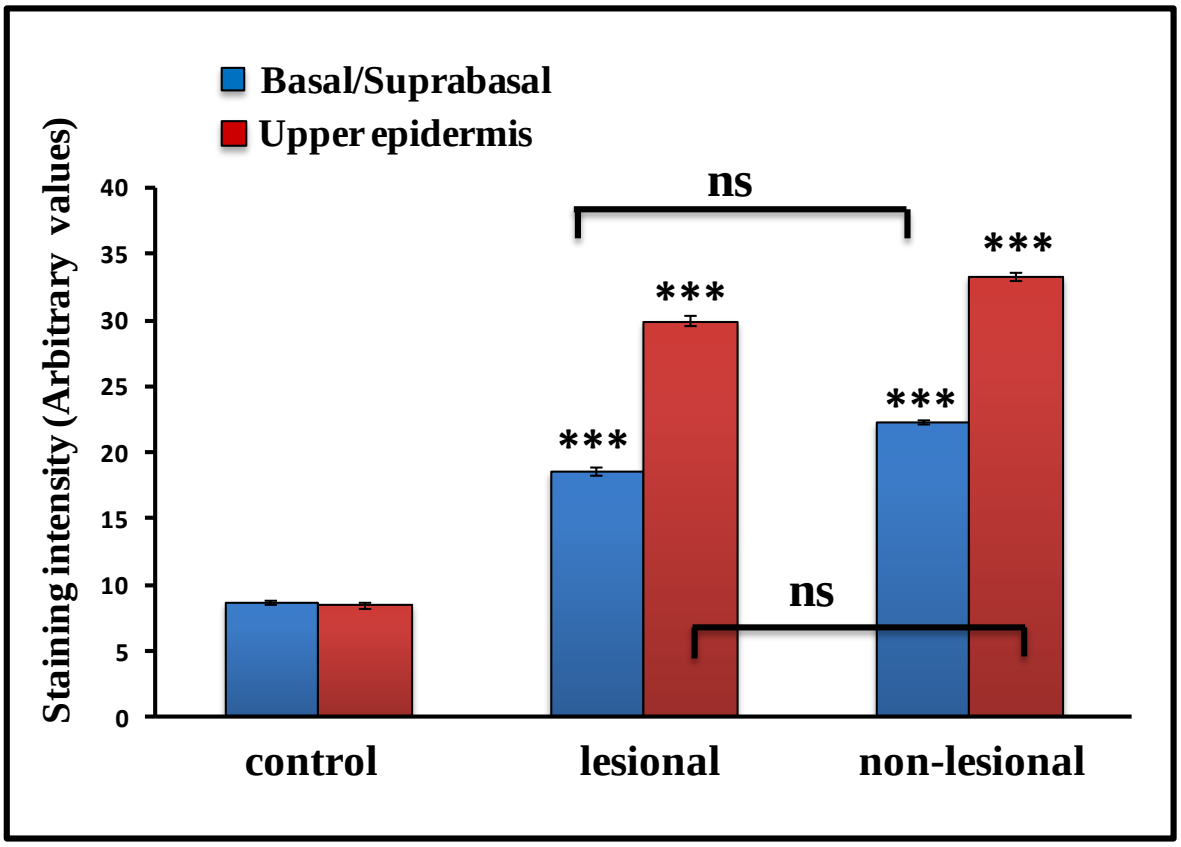

Figure 97: 5-nitro-tyrosine expression in MAL follows a gradient with an increase from basal / suprabasal layers to upper layers.

Image analysis of the average fluorescence intensity shows significantly increased levels of $ONOO^-$ in lesional (n=28: 4 intra-individual repeats, 7 pictures each) and non-lesional (n=28: 4 intra-individual repeats, 7 pictures each) skin of MAL. Plots are the mean of 4 different intra individual staining ± SE. (*** $p<0.001$, NS $p>0.05$)**.**

For comparison of nitration levels in MAL, in vitiligo and controls we analysed the ratio of 5-nitro-tyrosine in the entire epidermis. Our analysis reveals 0.5 fold higher nitrated 5-nitro-tyrosine expression in the basal layer of lesional skin of vitiligo compared to MAL. Hence, nitration levels are different between MAL and vitiligo **(Table 10)**.

Source	Epidermal layers	lesional	non-lesional
Vitiligo	basal	29.35	26.54
	upper	29.35	26.54
MAL	basal	18.63	21.00
	upper	29.97	33.32
Vitiligo / MAL ratio	basal	1.58	1.26
	upper	0.98	0.80

Table 10

5-nitro-tyrosine ratio in vitiligo and MAL patients' skin.

9.4.4.2. $ONOO^-$ does not affect SPARC in MAL

In contrast to our previously mentioned data in the vitiligo section of this thesis, double immuno-fluorescence staining of FITC-labelled SPARC and TRITC-labelled 5-nitro tyrosine in MAL shows only weak nitration of SPARC in nuclei, while nitration is almost absent in the cytosol and cell wall **(Figure 98)**. These data suggest that SPARC is not nitrated in MAL. This result is different to our observations in vitiligo. Moreover, it also may refer to the possible implication of SPARC in p53 inactivation and can introduce an explanation for the very different p53 / p21 expression in MAL.

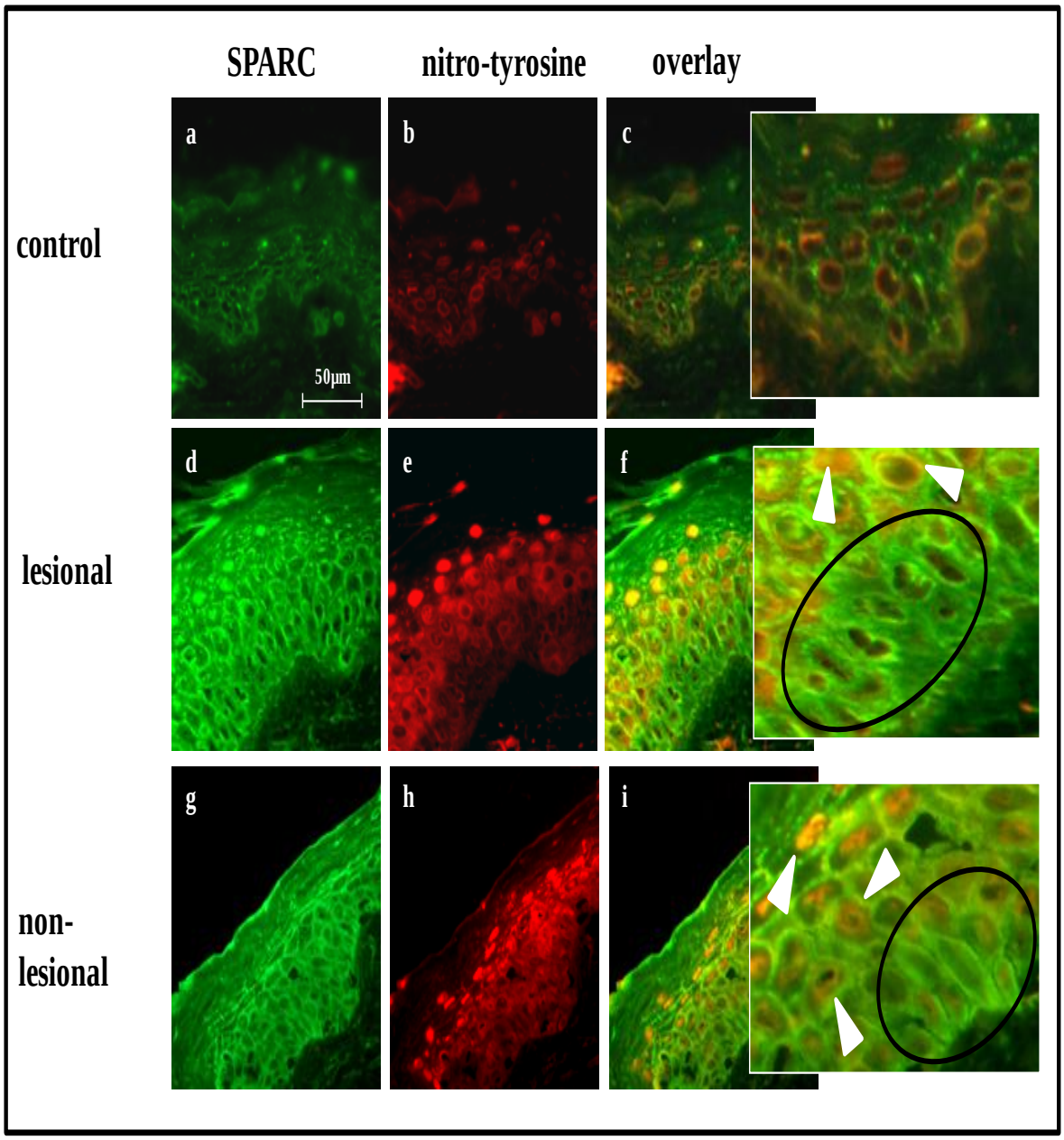

Figure 98: Positive nitration of SPARC in the upper epidermis of MAL.

Immuno-fluorescence reactivity of TRITC-labelled 5-nitro-tyrosine and FITC-labelled SPARC represent nitrated SPARC in MAL lesional (f) and non lesional (i) skin. Overlay of SPARC and nitrated tyrosine shows positive SPARC nitration in nuclei of the upper epidermis of both lesional and non-lesional skin (f, i and inserts) compared to healthy controls (c) (skin phototype III, Fitzpatrick classification). Basal and suprabasal layers show almost absent nitration. Scale bar 50µm. Magnification x 400.

9.5 VEGF-A expression in MAL

9.5.1 Increased VEGF-A expression in skin of MAL

It has been shown that VEGF-A expression is enhanced in keratinocytes after H_2O_2 and NO exposure (Brauchle, et al., 1996). At the same token, VEGF-A induces expression of SPARC in human vascular endothelial cells (Weninger, et al., 1996; Kato et al., 2001). This scenario appealed to us in the context of our interest. Therefore we decided to have a closer look at the expression of VEGF-A in skin of MAL.

Our *in situ* results show significantly higher expression of VEGF-A protein in lesional (d) and non-lesional (g) epidermis of MAL compared to control skin (a) **(Figure 99**). The data indicate again a gradient with increased expression in the upper layer of the skin. Image analysis of VEGF-A proves significantly increased protein expression in lesional (*** $p<0.001$, mean ± SE) and non-lesional (** $p<0.01$, mean ± SE) epidermis compared to controls **(Figure 100)**.

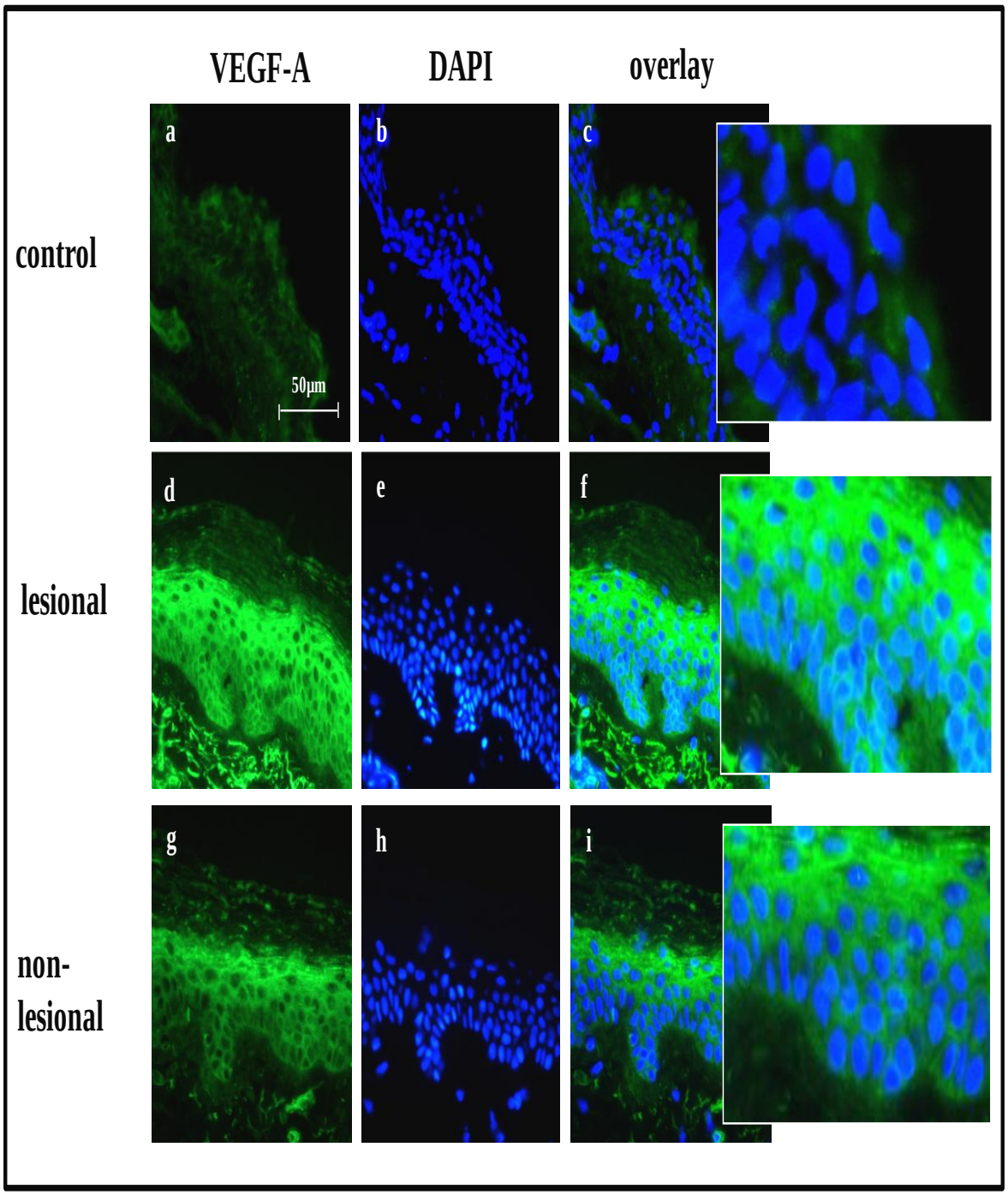

Figure 99: Strong expression of VEGF-A in MAL.

Immuno-reactivity staining (FITC-labelling in green) shows strong VEGF-A expression in both lesional (d) and non-lesional (g) skin of MAL compared to healthy control with skin phototype III (a). In non-lesional skin, SPARC expression appears to be weaker in basal / suprabasal layers. Scale bar 50µm. Magnification x 400.

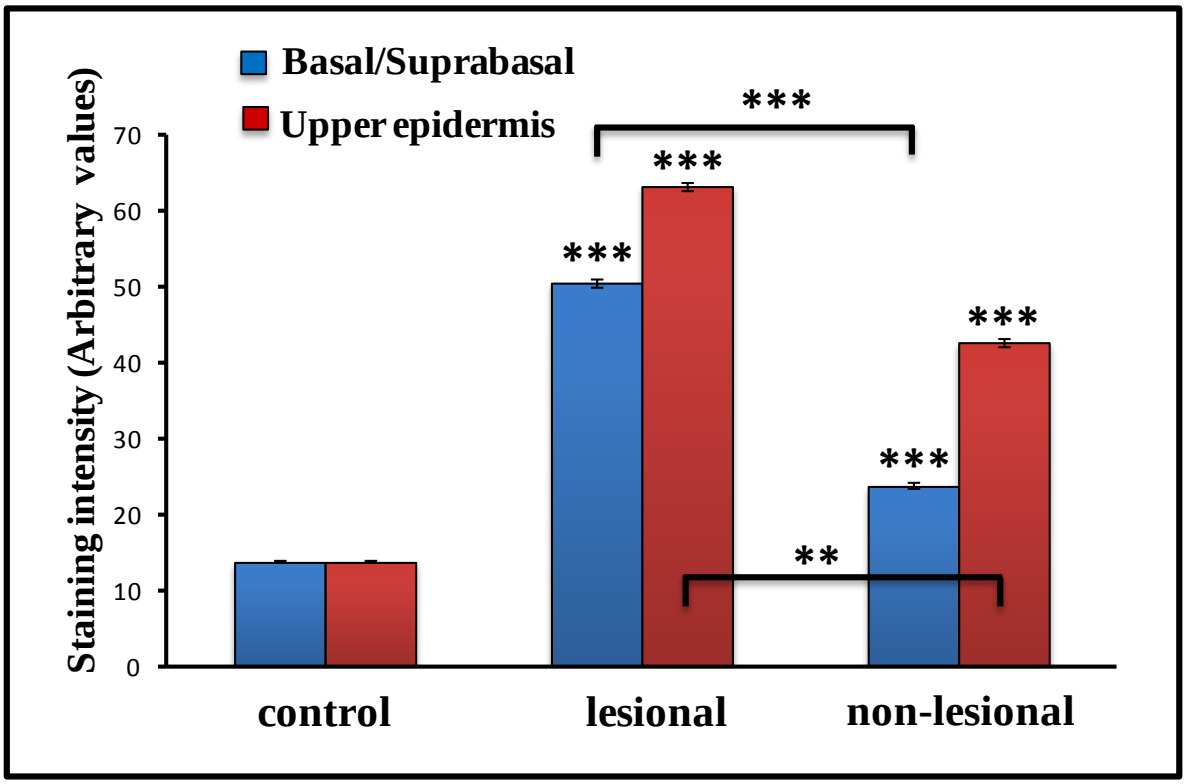

Figure 100: Significantly higher VEGF-A expression in MAL.

Image analysis of the average fluorescence intensity shows significantly increased expression of VEGF-A in lesional (n=28: 4 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin of MAL compared to controls (n=21: 3 individuals, 7 pictures each). Expression is significangtly stronger in the upper layer of the epidermis compared to basal / suprabasal layers. (Plots are mean ± SE) (** $p<0.01$, *** $p<0.001$).

9.5.2 Confirmation of VEGF-A up-regulation in MAL by Western blot analysis

To further support our observed increased VEGF-A expression in MAL as possible enhancer of SPARC expression, we used Western blot. The results reveal significantly higher VEGF-A levels in lesional and non-lesional skin of MAL compared to controls **(Figure 101a)**. Image analysis of VEGF-A protein bands in relation to loading control protein (GAPDH) confirms significantly increased VEGF-A levels in MAL compared to controls **(Figure 101b)**.

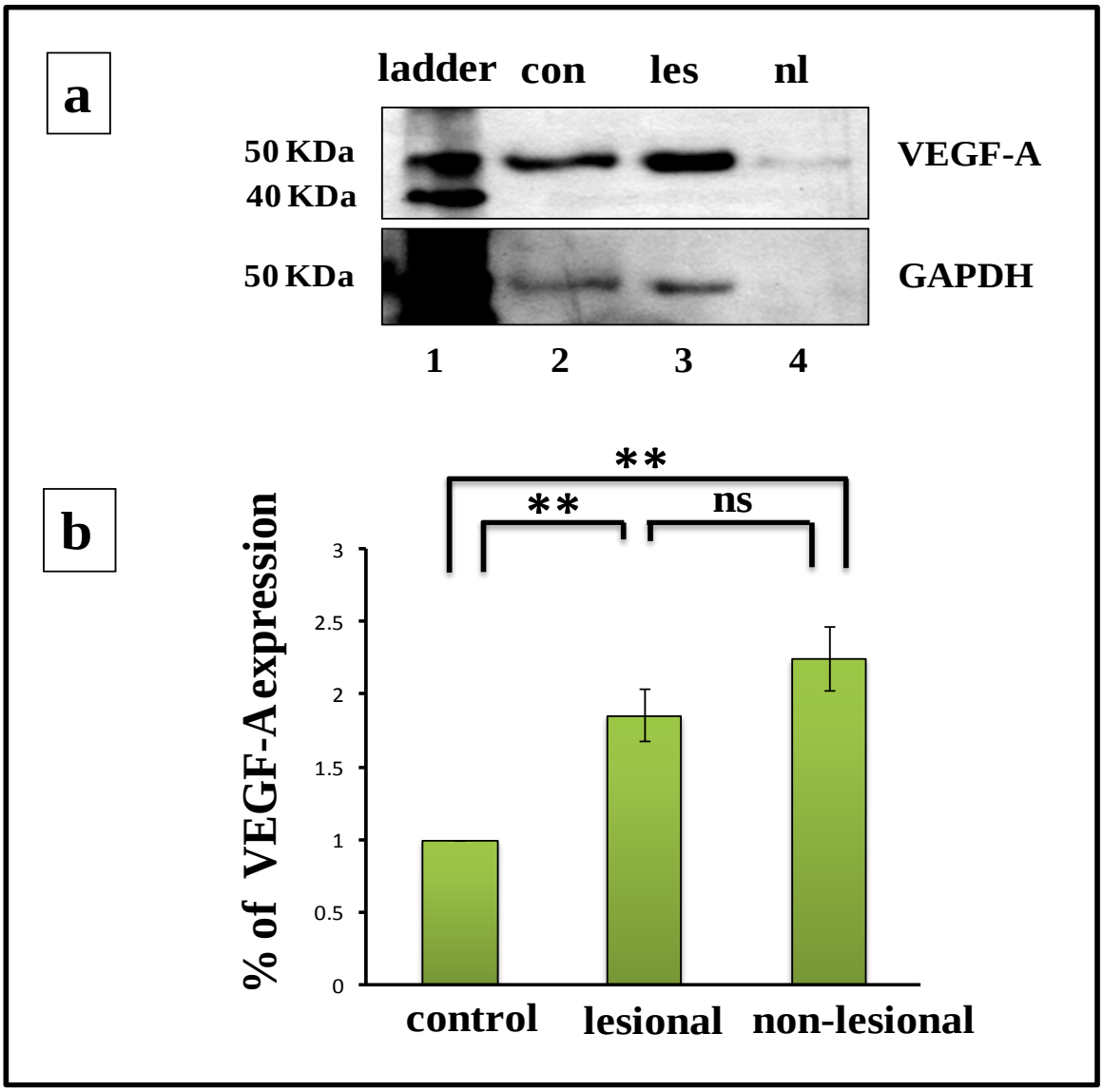

Figure 101: Significantly increased VEGF-A levels in MAL.

(a) Western blot shows increased VEGF-A in lesional and non-lesional skin of MAL compared to control skin. Lane1 protein ladder. lane 2 control, lanes 3-4 lesional and non-lesional skin extracts from MAL. GAPDH was used as loading control.

(**b) Quantification of VEGF-A levels.** Image analysis of the bands was used to quantify protein expression in lesional and non-lesional skin in relation to its expression in control skin. The result confirms increased VEGF-A expression in lesional and non-lesional skin of MAL compared to control skin (NS $p > 0.05$, ** $p<0.01$).

9.5.3 *In situ* VEGF-A expression in epidermal melanocytes

Overlay of FITC-labelled VEGF-A and TRITC-labelled NKI / beteb1 identifies very weak VEGF-A expression in melanocytes of non-lesional skin in MAL shows that the protein is expressed, while it is absent in the same cells of control skin **(Figure 102)**.

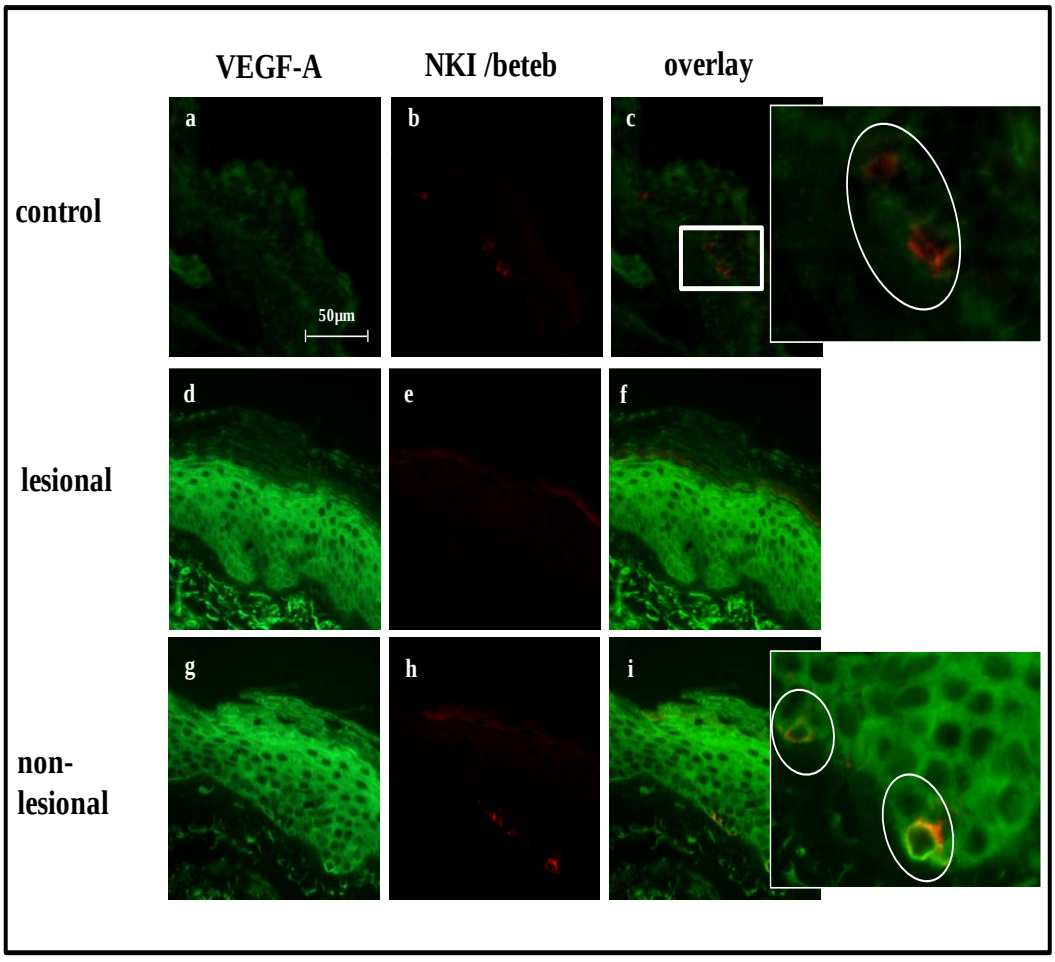

Figure 102: VEGF-A expression in epidermal melanocytes of MAL.

Immuno-reactivity staining (FITC-labelling, green) shows strong expression of VEGF-A in lesional (d) and non-lesional (g) skin of MAL compared to control skin (skin phototype III (a). Overlay of NKI / beteb1 positive epidermal melanocytes (TRITC-labelling) and FITC - labelled VEGF-A yields a weak VEGF-A expression in non-lesional skin of MAL (insert i), while control melanocytes completely lack expression (insert c). Magnification x 400. Scale bar 50µm.

9.5.4 VEGF-A nitration in MAL

Next we examined possible nitration of VEGF-A in MAL. The double immuno-fluorescence results shows almost absent co-localization of FITC- labelled VEGF-A and TRITC- labelled 5-nitro-tyrosine in the basal layer of both lesional and non-lesional skin of MAL **(Figure 103).** This result could support a functioning VEGF-A induced SPARC cascade in MAL.

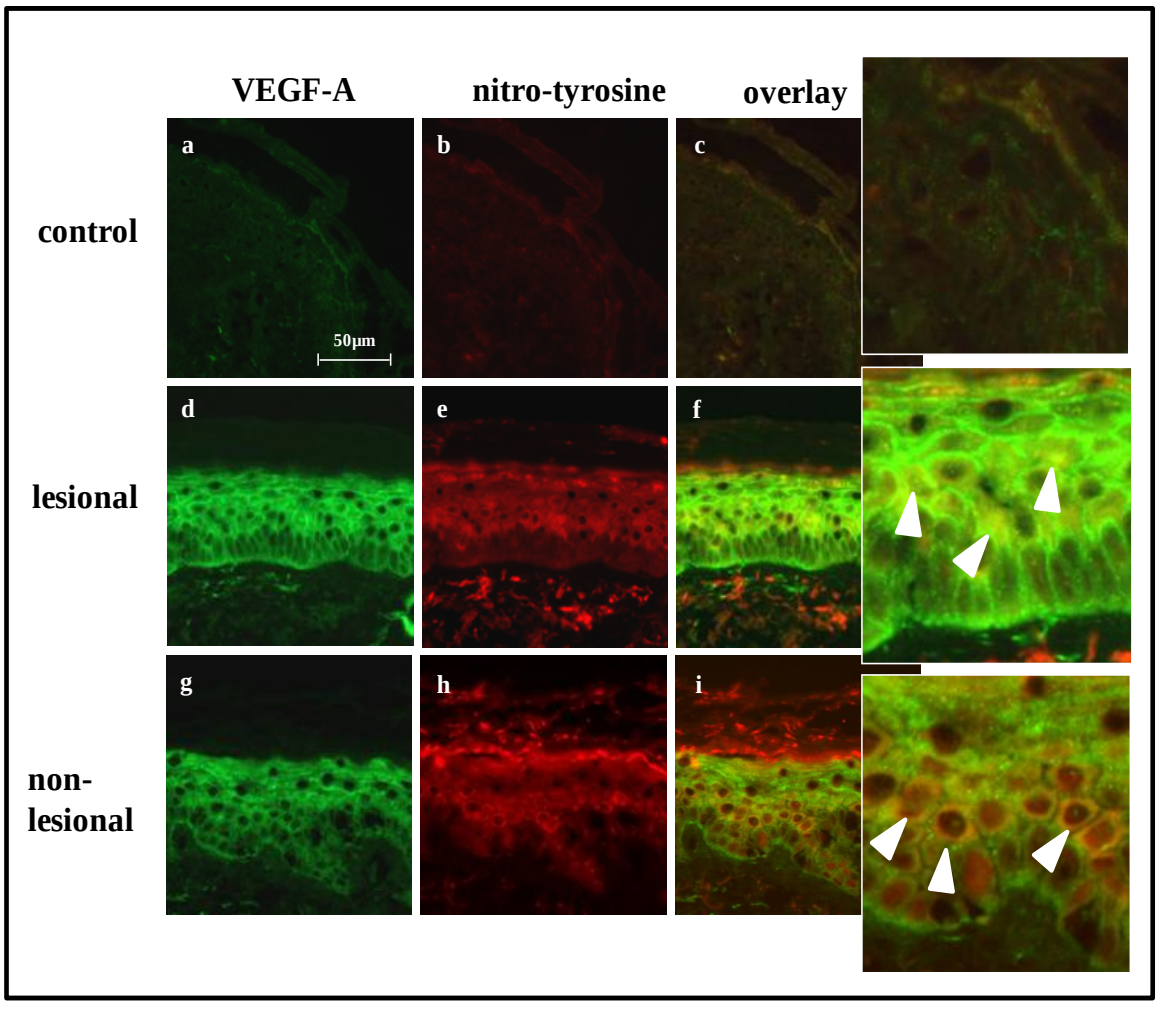

Figure 103: Evidence for nitration of VEGF-A in MAL.

Immuno-fluorescence reactivity of TRITC-labelled 5-nitro-tyrosine and FITC-labelled VEGF-A in MAL shows strong nitration in the nuclei of non-lesional (insert i, Δ) and some weaker expression in lesional epidermis (insert f) skin. Nitration is absent in nuclei and cytosol of the basal / suprabasal layer in MAL with some expression in the upper layer. Nitration seems stronger throughout the entire epidermis in non-lesional skin. Scale bar 50µm. Magnification x 400. The original experiment included analyses in control skin.

9.6 Evaluation of epidermal TGF-β1 expression in MAL

9.6.1 TGF-β1 expression corresponds to p21 expression in MAL

Given, that SPARC is functioning in MAL then p53 could be target to inactivation by this protein. This assumption is supported by the p21 expression in the upper epidermal compartment which was significantly higher compared to control skin, but lower compared to classical vitiligo. However, our MAL data demonstrated very strong p21 expression in the basal / suprabasal layer, while in classial vitiligo p21 is expressed throughout the epidermis. Here the question is, whether this p21 expression could be originating from p53-independent regulation. In this context it was known that TGF-β1 stimulates p21- production through a p53-independent mechanism (Rodeck et al., 1994; Krasagakis et al., 1999; Rodeck et al., 1999; Hoek et al., 2006). Our immuno-fluorescence show strong TGF-β1 expression in the basal / suprabasal layers in lesional and non-lesional epidermis of MAL compared to control skin) **(Figure 104)**. Overlay with DAPI suggests the presence of TGF-β1 in some, but not all nuclei **(Figure 104 (f, i)).** Image analysis of TGF-β1 confirms significantly increased protein expression in the basal / suprabasal layers of lesional and non-lesional skin compared to controls. The data are based on 3 intra-individual stainings of the same sample. Plots are mean ± SE. (*** $p<0.001$, NS $p>0.05$) **(Figure 105)**. TGF-β1 expression is completely absent in the upper epidermal layers of non-lesional skin. The predominant location of TGF-β1 in the basal layer corresponds with our *in situ* results from p21 expression in the same patient, involving two layers of the basal /suprabasal cells in lesional skin, while non-lesional and control skin only show expression in the basal layer **(Figure 104)**.

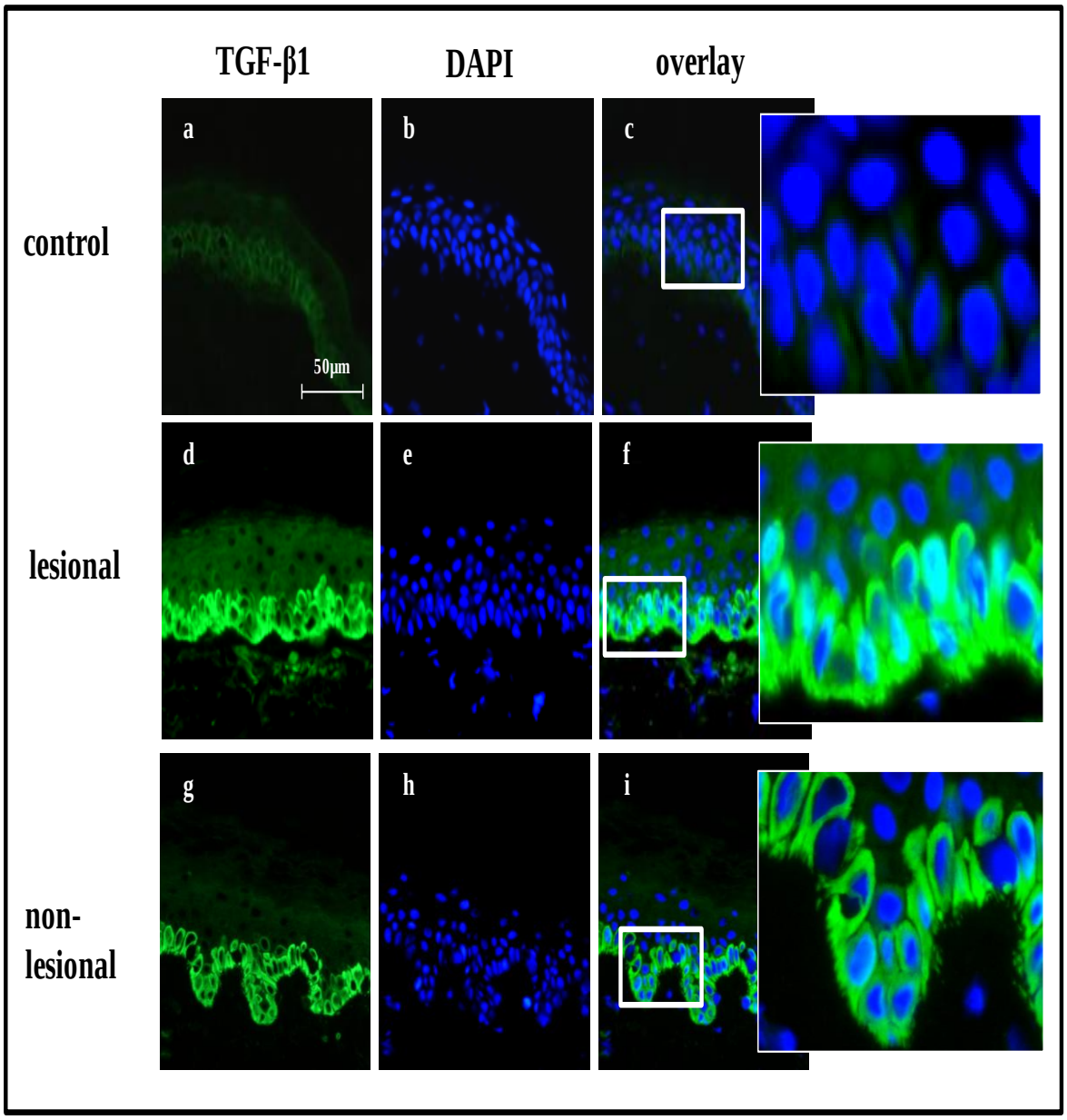

Figure 104: Increased cytosolic TGF-β1 expression in the basal / suprabasal layers of MAL.

FITC-labelling (green) of TGF-β1 expression reveals strong cytosolic expression in lesional (d) and non-lesional (g) skin of MAL compared to healthy controls (a). Overlay with DAPI suggests the presence of TGF-β1 in some nuclei of the basal layer in lesional and non-lesional skin (light blue). N.B. Expression of TGF-β1 extends to the suprabasal layers in lesional skin, while it is restricted to the basal layer in non- lesional and control skin. In MAL this result corresponds to the same distribution as p21, catalase, $p76^{MDM2}$, and MDM4 which all show reciprocal pattern compared to p53, VEGF-A and expression of $ONOO^-$. Scale bar 50µm. Magnification x400.

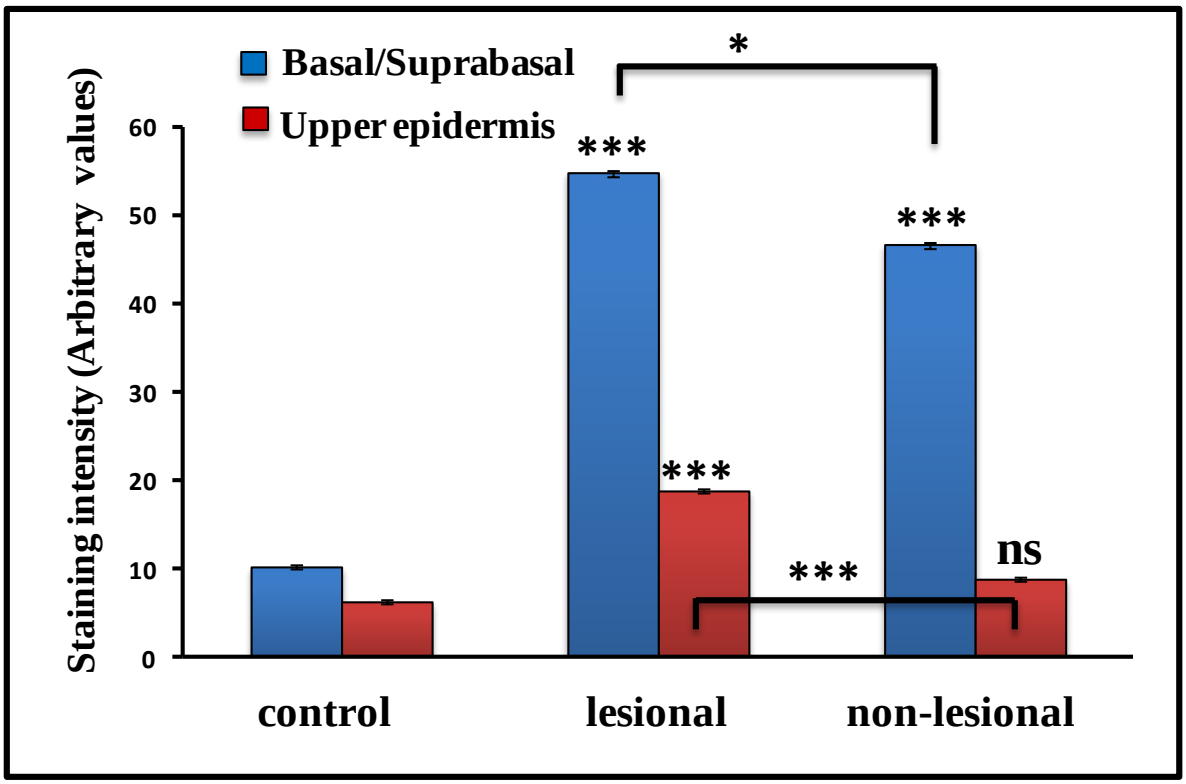

Figure 105: Significantly higher TGF-β1 expression in the basal / suprabasal layers of MAL.

Image analysis of the average fluorescence intensity yields significantly increased TGF-β1 levels in the basal/suprabasal layers of the epidermis in lesional (n=21: 3 intra-individual repeats, 7 pictures each) and non-lesional (n=21: 3 intra-individual repeats, 7 pictures each) skin of MAL compared to controls (n=24: 3 individuals, 8 pictures each). Expression is not significantly different between upper epidermis of non-lesional and control skin. Plots are the mean of 3 different intra-individual stainings ± SE. (*** $p<0.001$, NS $p>0.05$).

9.6.2 TGF-β1 is not expressed in epidermal melanocytes of MAL

Next, we looked, whether TGF-β1 is present in epidermal melanocytes. To address this question, we used double immuno-fluorescence with FITC-labelled TGF-β1 and TRITC-labelled NKI / beteb1. The *in situ* result showed no TGF-β1 expression in melanocytes in control and non-lesional epidermis of MAL **(Figure 106).**

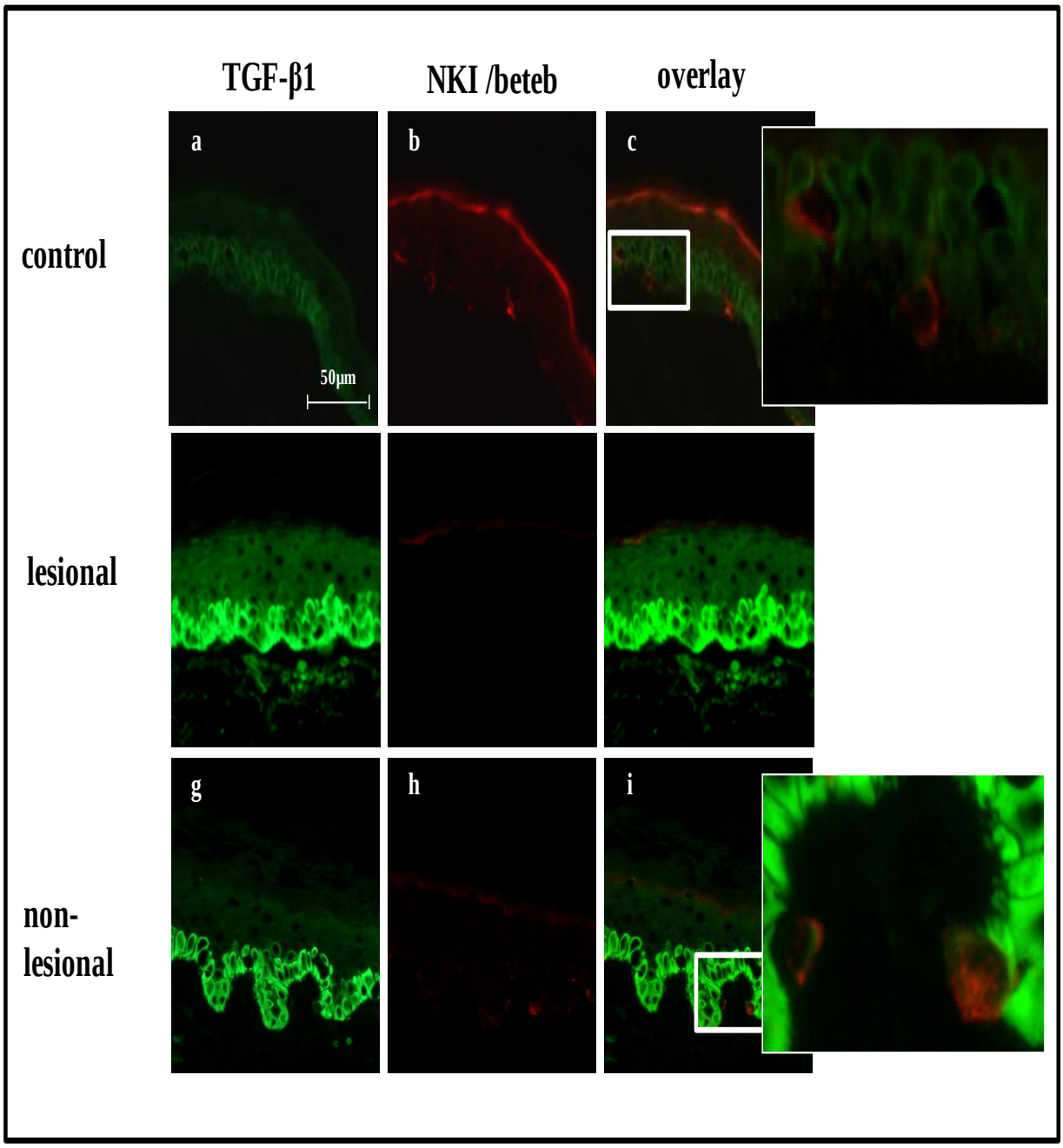

Figure 106: Absence of TGF-β1 expression in epidermal melanocytes of MAL.

Immuno-reactivity staining (FITC-labelling, green) shows high expression of TGF-β1 in lesional (d) and non-lesional (g) skin of MAL compared to healthy control (a). In this case our NKI / beteb1 labelled melanocytes are detached. Overlay of both chromophores show no sign for the presence of TGF-β1 in melanocytes. Scale bar 50µm. Magnification x400.

9.6.3 Western blot confirms significantly increased TGF-β1 expression in both lesional and non-lesional skin of MAL

In order to quantify protein expression, Western blot analysis was carried out. The results confirm increased TGF-β1 expression in both lesional and non-lesional skin of MAL compared to skin of healthy controls **(Figure 107a)**. Image analysis of TGF-β1 protein bands in relation to loading control protein (GAPDH) reveals significantly increased TGF-β1 expression in lesional and non-lesional skin of MAL **(Figure 107b)**.

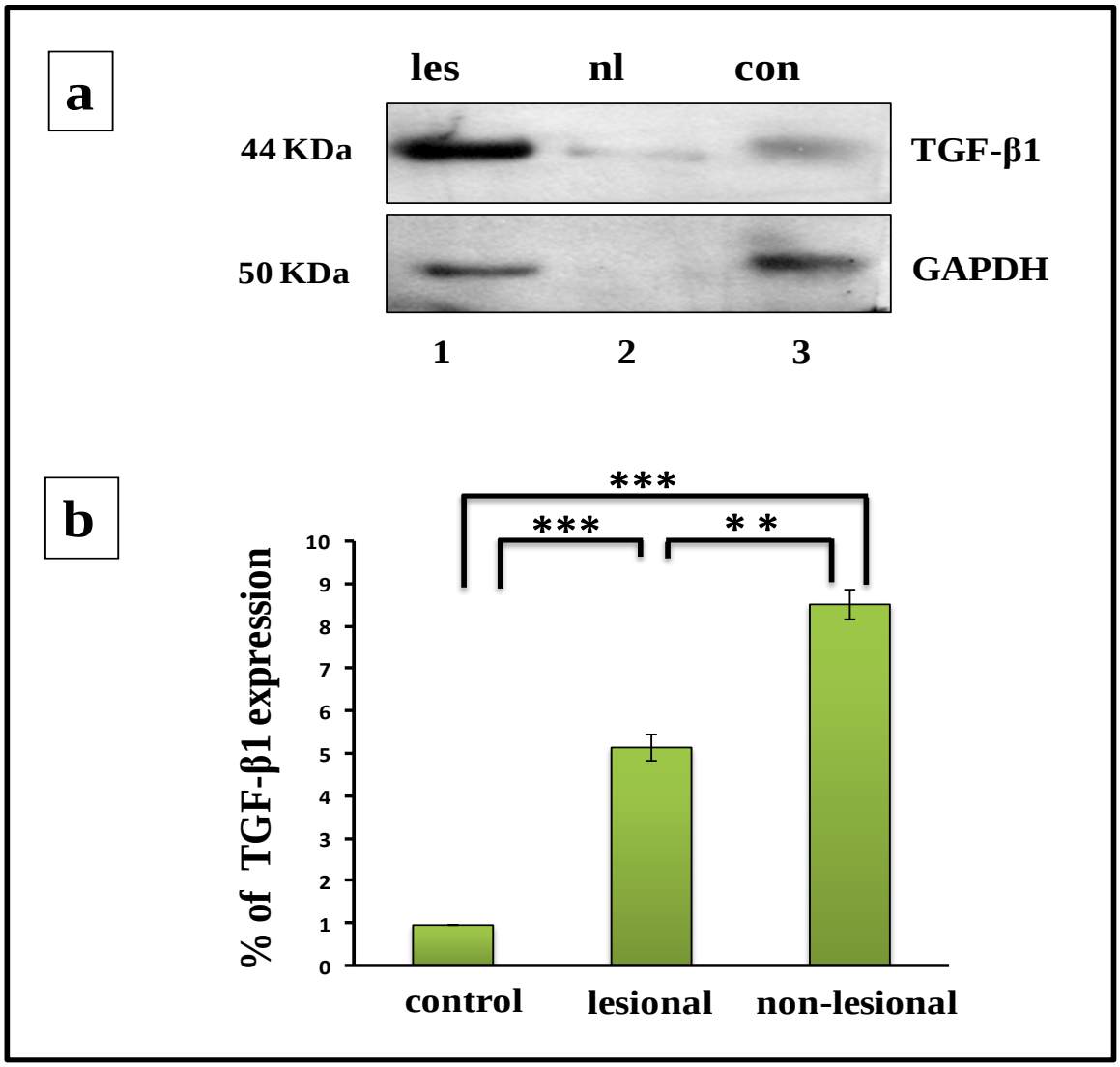

Figure 107: Significantly increased TGF-β1 expression in skin of MAL.

(a) Western blot of TGF-β1 shows expression of TGF-β1 protein in lesional and non-lesional skin of MAL compared to control skin. Lane 1 and 2 are lesional and non - lesional skin tissue extracts from the patient with MAL (respectively), lane 3 control. GAPDH was used as loading control.

(b) Quantification of the TGF-β1 bands. Image analysis was performed in relation to loading control protein (GAPDH). The result reveals significantly increased expression lesional and non- lesional skin of patients with MAL compared to normal controls (n=2). (Plots are mean ± SE) (*$p>0.05$, *** $p<0.001$).

9.6.4 TGF-β1 is weakly nitrated in non-lesional MAL

We then asked the question, whether $ONOO^-$ nitrates TGF-β1 in the epidermal compartment of MAL. The results show that TGF-β1 is weakly nitrated in non-lesional skin **(Figure 108)** but not in lesional and control skin. Based on these results, it is tempting to conclude that p21 expression in MAL is regulated by TGF-β1 in a p53 independent manner. This result is very different from classical vitiligo, where we found strong evidence for nitration of TGF-β1 in the basal / suprabasal layer in this entity.

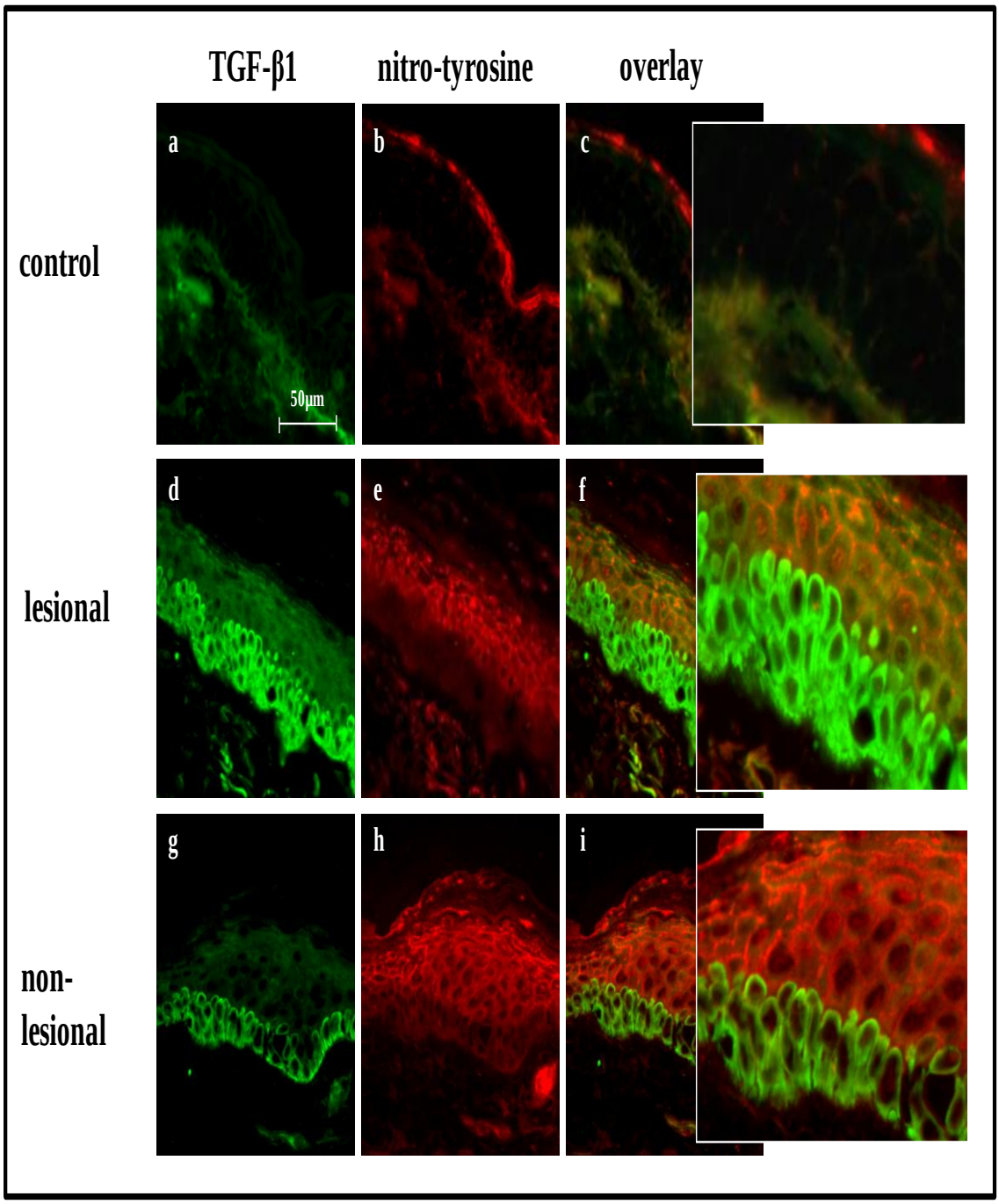

Figure 108: Weak nitration of TGF-β1 in non-lesional MAL.

Double immuno-fluorescence reactivity of TRITC-labelled 5-nitro-tyrosine and FITC-labelled TGF-β1 shows weak nitrated TGF-β1 in MAL in non-lesional skin corresponding to the presence of $ONOO^-$ in these layers. NB. There is no evidence for nitration of TGFβ1 in control skin. Scale bar 50µm. Magnification x 400.

10. Discussion

Malignant melanoma (MM) is associated with different forms of hypopigmentation which may either appear as regression in primary or metastatic melanoma, halo nevi around pre-existing melanocytic nevi, or melanoma associated leucoderma at sites distant from the primary tumour (Ortonne et al., 1978; Schallreuter et al., 1991a; Aisenbrey et al., 2003). In the literature this depigmentation has been documented with various names, including vitiligo. We here assigned the name melanoma associated leucoderma (MAL), as this term covers a precise clinical description of this postsurgical and / or immune induced depigmentation in association with malignant melanoma.

The aim of this thesis was, to find out, whether melanoma associated leucoderma (MAL) is a distinct clinical entity compared to classical vitiligo.

The development of “vitiligo” in melanoma patients is a well known. Still it presents a puzzling and poorly understood phenomenon. In this context it is important to recognize that melanoma is a cutaneous malignancy originating from a **proliferation of atypical melanocytes**, while classical vitiligo is characterised by hypopigmented / depigmented skin lesions with a **loss of functional epidermal melanocytes** (Schallreuter et al. 2008a, Tobin et al., 2000; Schallreuter, 2014; Quaglino et al., 2010).

In 1991, Schallreuter and colleagues studied this hypopigmentation phenomenon in 623 patients with cutaneous melanoma. Eleven cases had classical vitiligo at least 2 years before diagnosis of melanoma (n=11), while others (n=11) developed melanoma associated depigmentation after surgery of their primary and / or metastatic melanoma (Schallreuter et al., 1991a). In a prospective cohort study from a single institution vitiligo was documented in 83 of 2954 melanoma patients, yielding in turn a

cumulative incidence of 2.8% compared to 3.7% in the Hamburg study and data from 4.1% according to Bystryn and colleagues (Bystrin et al.,1987; Schallreuter et al 1991; Quaglino et al., 2010). In the Italian study the incidence of "vitiligo" was before 0.6% and after melanoma 2.2%. Hence, these numbers are similar to normal subjects considering a worldwide prevalence ranging between 0.5-1.5% (Krüger et al., 2012). Quaglino and co-workers strongly argued that "melanoma-associated vitiligo" is a distinct clinical entity, separate from vitiligo vulgaris. Moreover, in their study showed a significantly higher percentage of autoimmune diseases in the "vitiligo" patients and melanoma (7 of 83) compared to melanoma patients without vitiligo (80 of 2871) ($p = 0.004$). Therefore these authors suggested a distinct "vitiligo" for a subgroup of patients with a high prevalence of immune-mediated diseases in association with a favourable tumour prognosis (Quaglino et al., 2010). One aim of this thesis was to characterize and compare MAL with classical vitiligo. For this purpose we studied epidermal catalase expression, as this protein is one reliable and established biomarker for H_2O_2-mediated stress in vitiligo (Schallreuter, 2014). In addition, we used a panel of epidermal proteins around the regulation of p53, including p21, p76 ^{MDM2}, MDM4, MDM4phospho, SPARC, VEGF-A, TGF-β1. Moreover, we included evaluation on nitration via $ONOO^-$ levels, as this radical is an additional biomarker for oxidative / nitrative stress in classical vitiligo (Schallreuter et al 2003; Salem et al., 2009).

Presence of epidermal H_2O_2 and some oxidation products in MAL

Our first approach was *in vivo* evaluation of epidermal H_2O_2 levels with MAL using *in vivo* FT-Raman spectroscopy. To the best of our knowledge, our results identified for the first time the presence of H_2O_2 accumulation in MAL together with some oxidation products, including 5-OH-tryptophane, methionine sulfoxide and formyl kynurenine /

kynurenine in MAL **(Figure 71)** (Rokos et al., 2004, Schallreuter et al., 2012a). Based on this result we can conclude that classical vitiligo and MAL show the same results for epidermal oxidative stress.

Despite confirmation of H_2O_2 accumulation and oxidation of some assigned amino acids, epidermal catalase expression was still present, but lower in lesional and non-lesional skin of MAL compared to control skin. Moreover, protein expression followed a gradient with significantly higher levels in the basal / suprabasal layers than in the upper layers **(Figure 72).** These findings differ from classical vitiligo, where catalase expression is lacking this gradient and expression is significantly lower or even absent (**Figure 17**) (Schallreuter etal., 1991; Salem et al., 2009, Schallreuter, 2014). Interestingly, our data are in agreement with a previous study, which documented decreased epidermal catalase expression in normal skin of patients with melanoma (Sander et al., 2003).

Here it is noteworthy that catalase is highly expressed in epidermal melanocytes in healthy control skin, while the enzyme is absent in non-lesional melanocytes of MAL and in non-lesional classical vitiligo **(Figures 17, 74)**. This result is supported with data from Maresca and colleagues, who observed in melanocytes a correlation between catalase levels and melanogenesis (Maresca et al. 2007).

Given, that $ONOO^-$ levels are significantly increased in both lesional and non-lesional skin of patients with classical vitiligo (Salem et al., 2009 and here in this thesis), we wanted to know, whether this free radical could be a player in MAL. Our immuno-fluorescence labelling of 5-nitro-tyrosine, as the foot print of $ONOO^-$, shows significantly higher expression in the upper epidermal layers of MAL with almost absent expression in the basal / suprabasal layers **(Figure 96).** This result is very different compared to classical vitiligo, where the distribution of 5-nitro-tyrosine

indicates nitration throughout the entire epidermal compartment of lesional and non-lesional skin **(Figure 50).**

In order to compare nitration levels between vitiligo and MAL, ratios of $ONOO^-$ expression throughout the epidermis were calculated. This analysis revealed higher nitration levels in the basal / suprabasal layers of classical vitiligo compared to MAL **(Table 6).**

Source	Epidermal layers	lesional	non-lesional
Vitiligo	basal	29.35	26.54
	upper	29.35	26.54
MAL	basal	18.63	21.00
	upper	29.97	33.32
Vitiligo / MAL ratio	basal	1.58	1.26
	upper	0.98	0.80

Table 10

Comparison of epidermal $ONOO^-$ - levels in classical vitiligo and MAL (shown earlier).

To sum up, our immuno-fluorescence data show in MAL considerable epidermal oxidative / nitrative stress expression. Importantly, levels seem to be higher in the upper (differentiating) part of the epidermis, while the basal / suprabasal cells, forming the proliferation zone, are more protected against oxidative / nitrative intervention. *In vivo* FT-Raman results are not different between classical vitiligo and MAL. However, as catalase expression is present in MAL, it was tempting to invoke a more efficient

turnover of H_2O_2 with less $ONOO^-$ formation. In this context it is of interest that the epidermal proliferation zone is better prortected compared to the differentiation zone in the same compartment.

Detection of a distinct p53 / p21 cascade in MAL

After confirmation of up-regulated functioning epidermal wild type p53 levels in part I of this thesis (Schallreuter et al., 2003; Salem et al., 2009), together with the distinct different catalase expression patterns in classical vitiligo and MAL, we compared the p53 cascade in lesional and non-lesional skin of both. In MAL we found significantly up-regulated p53 levels in the upper epidermis with lower expression in the basal / suprabasal layers **(Figures 78, 81)**. These low p53 levels in basal keratinocytes were also present in melanocytes of non-lesional skin with almost absent p53 expression. Our *in situ* results of p21 expression in MAL revealed a reciprocal pattern compared to p53 expression. Expression levels of p53 and p21 are differing significantly from classical vitiligo, where both are increased throughout the entire epidermis **(Figures 82, 84, 85).** Under *in situ* conditions melanocytes show weak p53 and p21 expression in non-lesional skin of MAL. This result is different to classical vitiligo, as those melanocytes express high p53 and p21 levels in non-lesional skin **(Figures 23, 27, 77, 82).**

High p53 levels have been reported in melanoma (Yamamoto and Takahashi, 1993; Montano et al., 1994; Sparrow et al., 1995; Zerp et al., 1999; Rass et al., 2001; Soussi and Beroud, 2001; Soto et al., 2005; Gwosdz et al., 2006; Li et al., 2006). Our own data show significantly up-regulated p53 in the upper layers of MAL skin, regardless of lesional or non-lesional origin. Here it is tempting to propose, that the entire skin of patients with MAL may be regulated by a reciprocal presence of p53 and / or p21 interaction in the epidermis. Our MAL data are supporting the suggestion by Avery-

Kiejda and colleagues, who proposed loss in control of p53 target genes, including p21 in melanoma (Avery-Kiejda et al., 2011).

This reciprocal p53 / p21 expression pattern raises some questions.

One major question seems to be, whether up-regulated p53 is functional in MAL. The presence of higher p21 expression in the upper layers of MAL skin may suggest a functional p53 for induction of p21 expression in the differentiation zone of the epidermis **(Figures 75, 78),** This assumption would be supported via the presence of H_2O_2 / $ONOO^-$ leading to enhancement of p53-DNA - binding capacity (Salem et al., 2009). The next question is: How is the scenario in the basal / suprabasal layers where proliferation is taking place?

Could the presence of p76^{MDM2} explain p53 accumulation in MAL?

As said above, the MDM2 family plays an important role in p53 regulation (Haupt et al., 1997; Honda and Yasuda, 2000; Kubbutat et al., 1997; Oliner et al., 1993; Perry et al., 2000; Giglio et al., 2010). Briefly, p76^{MDM2} has a positive regulatory function towards p53 via antagonising p53-p90^{MDM2} binding, preventing in turn p53 ubiquitination (Perry et al., 2000; Giglio et al., 2010). In addition, p76^{MDM2} binds to *p53* mRNA and promotes translation (Naski et al., 2009).

In this context it is of interest that significantly up-regulated epidermal p76^{MDM2} splice variant has been documented in classical vitiligo (Salem et al., 2009). This result was confirmed in this thesis / partI. Therefore our next interest was, getting a closer look into p53 degradation.

Our immuno-fluorescence and Western blot data showed up-regulated p76^{MDM2} in lesional skin of MAL with high levels in the basal proliferation zone and significantly less expression in the upper differentiation layers of the epidermis. Hence, p76^{MDM2}

expression correlates with a reciprocal p53 expression **(Figure 83).** These data could point to a lack of functioning $p76^{MDM2}$ in control of p53 degradation in the proliferation zone of MAL. Moreover, here it becomes of interest that the p53 gradient in MAL correlates with a reciprocal pattern of catalase and p21 expression. With other words, the proliferation zone is more efficient in H_2O_2-reduction due to significantly up-regulated catalase, while H_2O_2 levels may be higher in the upper layers, as catalase expression is significantly lower in this upper differentiation zone. This assumption would be supported by the fact, that H_2O_2 induces p53 (Vile, 1997; Xie et al., 1999).

Importantly, our MAL results point to a distinct difference to our classical vitiligo data (Salem et al., 2009 and in part I of this thesis), where distribution of p53 and $p76^{MDM2}$ protein expression spans the entire epidermis without showing significant differences in expression levels between any layers.

MDM4 in MAL is phosphorylated and therefore may not be involved in p53 regulation

MDM4 is exercising controversial roles in control of p53. Some studies support a positive regulatory role for MDM4 towards p53 through antagonising p53 / $p90^{MDM2}$ binding (Sharp et al., 1999; Jackson and Berberich, 2000; Stad et al., 2000). Other authors suggested cooperation of $p90^{MDM2}$ and MDM4 after heterodimerization, influencing in turn p53 levels and function (Kawai et al., 2007).

Our *in situ* immuno-fluorescence results show significantly higher MDM4 expression in lesional and non-lesional skin of MAL with more pronounced expression in the basal / suprabasal layers than in the upper layers **(Figure 86).** Taking into consideration the positive role of MDM4 towards p53 levels, we would rather have expected high p53 levels in the basal / suprabasal layers and not in the upper layers. However, our p53

data showed a reciprocal expression gradient in MAL. Hence, a positive influence of MDM4 expression on p53 should be absent. Bearing in mind the negative action of MDM4, our results rather imply MDM4 in control of p53 in MAL. These data are supported by a proposed inactivation of MDM4 in response to phosphorylation (Jin et al., 2006). Importantly, this action can cause retention of the protein in the cytoplasm away from $p90^{MDM}$. Our immuno-fluorescence labelling exhibited a high degree of MDM4-phosphorylation throughout the entire epidermis **(Figure 89).** Calculation of MDM4phospho / MDM4 ratio in control skin in addition to lesional and non-lesional skin of MAL revealed total phosphorylation of the protein in MAL **(Table9).** This result favours exclusion of MDM4 in control of p53 ubiqitination in MAL, while in classical vitiligo we showed partial phosphorylation of MDM4 **(Table5).**

Our overall data of MDM4 in MAL are different from our classical vitiligo results, suggesting impaired MDM4 function by the action of $p76^{MDM2}$ which may prevent heterodimerization of MDM4 with $p90^{MDM2}$ and consequently ubiquitination of p53. Another mechanism could be activation of p53 via inhibiting the binding of MDM4 and / or $p90^{MDM2}$ to p53. Moreover, this result could point to the probability that another yet unknown protein may be in control of this different p53 expression in MAL.

Protein	Epidermal layer	control	lesional	non-lesional
MDM4	basal	10.76	57.27	30.82
	upper	10.42	37.02	29.02
MDM4phospho	basal	18.7	68.99	58.24
	upper	18.7	68.99	58.25
MDM4phosph/MDM4	basal	1.74	1.20	1.89
	upper	1.79	1.86	2.01

Table 9

MDM4phospho-MDM4 immuno-fluorescence ratio in the basal / suprabasal and upper layer of MAL.

SPARC nitration - can it explain the different p53 expression pattern between vitiligo and MAL?

In order to shed more light on the reason behind our observed p53 expression gradient in MAL and based on data of Fenouille and colleagues, who reported a negative role of SPARC over-expression towards p53 levels and activity (Fenouille et al., 2011a;b), it was tempting to study the expression of this protein in our case of MAL. Similar to our classical vitiligo results presented in part I of this thesis, the epidermis of MAL holds significant up-regulated SPARC expression based on immuno-fluorescence and Western blot techniques **(Figures 92, 94).** The expression is distributed throughout the entire epidermis. These results together with our catalase data may refer to direct up-regulation of SPARC in MAL via increased H_2O_2 levels in the epidermis. A second possibility could be based on H_2O_2 mediated increase of SPARC levels via VEGF-A. This idea is supported by documented VEGF-A increase in response to H_2O_2 (Brauchle, et al., 1996). A possible role for VEGF-A in regulation of SPARC stems from the

observation that this protein is enhancing SPARC expression in human vascular endothelial cells (Kato et al., 2001; Weninger, et al., 1996).

The up-regulated SPARC throughout the entire epidermis of MAL in association with low p53 expression in the basal / suprabasal layers raised the question about possible inactivation of SPARC in the upper layers of the epidermis. As we showed the presence of $ONOO^-$ in MAL, following a gradient from basal / suprabasal layers to the upper epidermis, we considered the possibility of SPARC inactivation by $ONOO^-$ mediated nitration as observed in the case of classical vitiligo. Our data showed almost absence of SPARC nitration in basal / suprabasal layers in lesional skin with very weak nitration in the same layers of non-lesional skin in MAL **(Figure 98).** Interestingly, nitrated SPARC is present in nuclei of the upper layers of both lesional and non-lesional skin **(Figure 98).** These data support our observed, low p53 expression in basal / suprabasal layers as well as its high levels in the upper layers of MAL skin.

Based on these data, it is tempting to suggest that SPARC protein is inactivated in the upper layers of MAL epidermis. This scenario would be the same as in classical vitiligo and it is very likely caused by ROS / RNS-mediated stress. However, in the basal / suprabasal layers of MAL the situation is not the same. It appears completely different from classical vitiligo. Here SPARC is not nitrated in association with low p53. This result may suggest that SPARC is still functioning, fostering in turn p53 inactivation / degradation.

Could increased VEGF-A support up-regulated SPARC protein expression in MAL?

VEGF-A is expressed in both lesional and non-lesional skin of MAL **(Figures 99, 104).** The result shows less expression in the basal / suprabasal layers compared to the upper part of the epidermis. In order to investigate a possible role in control of SPARC up-regulation via VEGF-A in the presence of H_2O_2 / $ONOO^-$ in the upper layers of MAL, we used VEGF-A co-localization with 5-nitro-tyrosine. Our data showed very little co-localization in both lesional and non lesional skin **(Figure 103).** These data could support a possible role of VEGF-A induction of SPARC as reported in human vascular endothelial cells (Kato et al., 2001; Weninger, et al., 1996).

In this context it is of interest that up-regulated VEGF-A in melanoma coincides with induction of new blood vessel formation (Brauchle, et al., 1996; Claffey et al., 1996; Graells et al., 2004; Srivastava et al., 2003). Our data on up-regulated VEGF-A expression in lesional and non-lesional skin of MAL may refer to the possibility that VEGF-A levels increase at the entire integument of patients with melanoma and not only *in loco* of the tumour.

Can TGF-β1 explain p53-independent expression of p21 in MAL?

To get a better understanding of the reasons behind our observed reciprocal pattern in p53 / p21 expression in MAL, it was tempting to study the expression of TGF-β1. In this context it is of note that this protein can enhance p21 in a p53 independent manner (Datto et al., 1995; Li et al., 1995; Kim et al., 2006). Our immuno-fluorescence and Western blot results showed significantly up-regulated TGF-β1 in the basal / suprabasal layers in lesional and non-lesional skin of MAL, while expression was much lower in the upper epidermis. The result was congruent with p21 protein expression **(Figures**

104, 107). These overall data may suggest the capability of TGF-β1 in compensating for active p53 shortage and / or inactivity in the basal / suprabasal layers. The increase in TGF-β1 expression in basal / suprabasal layers could be due to the up-regulation of functional and non-nitrated SPARC. This mechanism has been shown in rat mesangial cells *in vitro* and *in vivo*, where TGF-β1 increased in response to elevation of SPARC levels (Bassuk et al., 2000). TGF-β1 is not nitrated in the basal / suprabasal layers of MAL, while the upper layers seem to be nitrated **(Figure 108).** These results favour TGF-β1-triggered p21 expression in the basal / suprabasal layers in MAL rather than p53 induced p21 expression as observed in classical vitiligo. It also points out to another difference between MAL and classical vitiligo. In this context, our data in classical vitiligo revealed totally non-functional nitrated TGF-β1 in both lesional and non-lesional skin. This is very different in MAL.

These overall data in MAL may propose a a link between H_2O_2 / $ONOO^-$ and depigmentation via inducing VEGF-A / SPARC / TGF-β1 cascade but this hypothesis need more investigations as well as samples to be proved **(Figure 109).** Our prospects are based on the facts that TGF-β1 is known as a promoter for hypopigmentation. It affects melanogenic proteins negatively as it is able to degrade or inactivate the tyrosinase enzyme. Moreover, it also down-regulates the production of MITF, TRP-1 and TRP-2 leading to disturbances in melanosomal maturation and inhibition of melanogenesis resulting in hypopigmentation (Martínez-Esparza et al., 1997; Martínez-Esparza et al., 2001; Kim et al., 2003).

Taken together, based on our data, we can conclude that MAL is a distinct different entity compared to classical vitiligo. The drawback of this work is that we only had one case four extensive study of MAL. However, as we found so many differences

compared to classical vitiligo, we still feel that this work is valid and provides a major contribution to the understanding of this peculiar depigmentation.

As MAL has been implicated in better prognosis of melanoma survival, it is noteworthy that our study patient presented at time of first diagnosis in 8/2008 an ulcerated nodule in a superficial spreading melanoma, arising in a congenital naevus. The histology confirmed a Clark level IV and a Breslow thickness of 4.67 mm. Importantly, the patient did not undergo lymphnodectomy. He developed the first signs of depigmentation in 2009. Until 4/2015 he has not developed any signs of metatstases. Importantly, our patient had no other immune diseases. Therefore we cannot connect his individual survival time to the proposed better overall outcome for this distinct patient group as suggested by Quaglino and colleagues (Quaglino et al., 2010). In order to validate the influence of immune diseases in the context of our investigation, more patients are needed with characterised MAL.

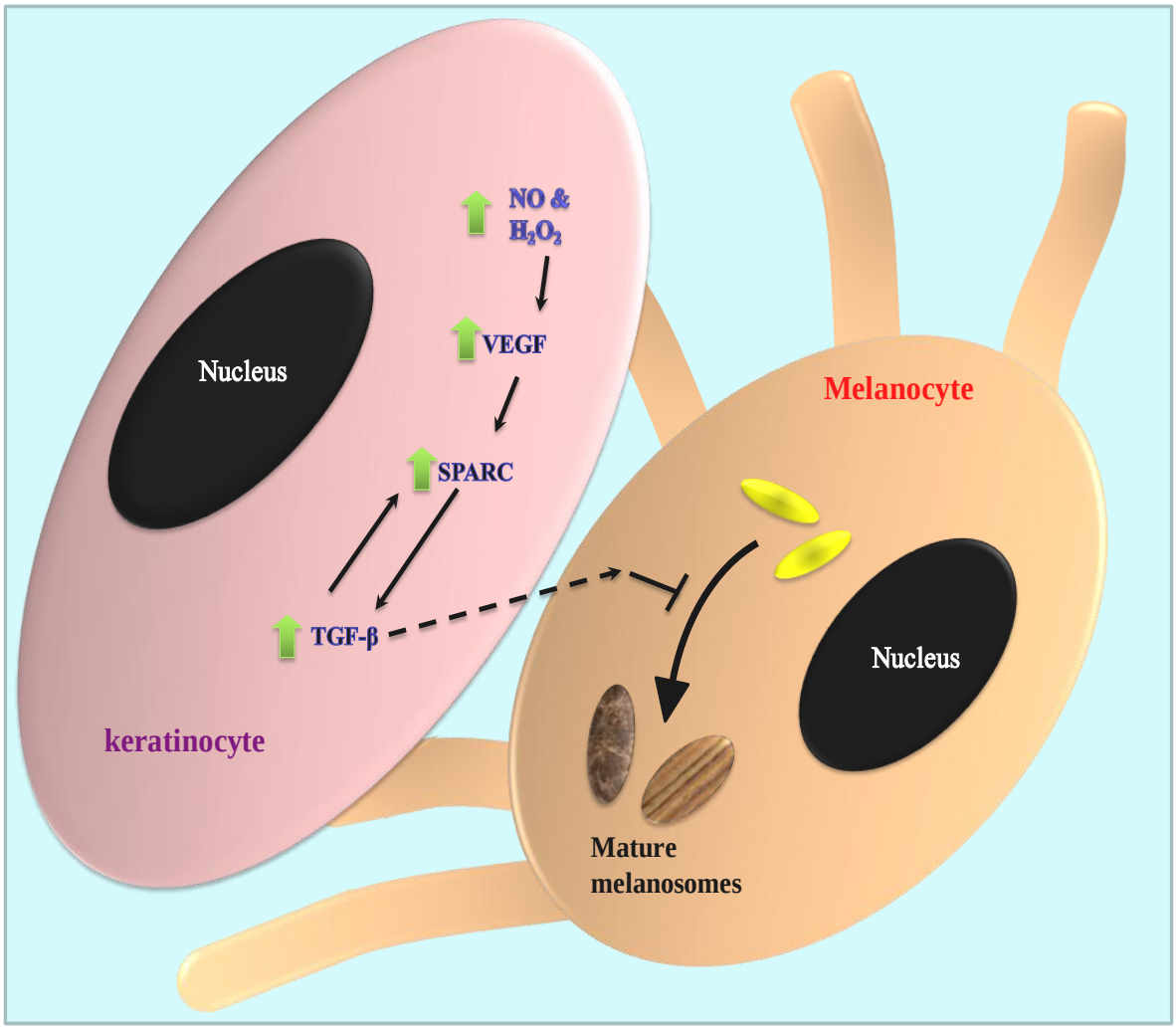

Figure 109: Hypothetical pathway involved in depigmentation in MAL.

A scheme summarising the possible role of H_2O_2 / $ONOO^-$ in promoting melanoma associated leucoderma (MAL) via induction of TGF-β1 expression; the hypopigmentaion promoter.

11. Conclusion

Based on recent data and our novel results, we can conclude that the entire epidermal homeostasis in vitiligo is affected in the disease process. (PART I).

1. Based on recent data together with our novel data presented herein, we can extend strong evidence that the **entire epidermal compartment, regardless , whether it is depigmented (lesional) or normal pigmented (non-lesional) is part of the depigmentation process in vitiligo.** We can conclude that all epidermal keratinocytes spanning from proliferation up to differentiation, are active players in the disease process. Hence, this disease does not only include absence of functioning melanocytes. **Vitiligo is a disease affecting the entire epidermal unit**.
2. We have confirmed the presence of oxidative and nitrative stress overload affecting the entire epidermis. Whether this is the cause or consequence of the disease, still needs to be shown. However, to date epidermal H_2O_2 accumulation together with its consequences is a fact in the pathogenesis of vitiligo.
3. Although patients with vitiligo lack partially protection against UVR due to absence of melanin in their depigmented skin, in addition to massive ROS / RNS-mediated oxidative / nitrative stress in their entire epidermal compartment, they do not present a significant elevated risk for development of non-melanoma skin cancer (NMSC) and photo-damage despite signs for increased DNA-

damage in the skin and plasma(Calanchini-Postizzi and Frenk, 1987; Schallreuter et al 2002; Salem eta., 2009; Teulings et al., 2013)

Our data confirmed previous results from our group (Salem et al 2009). The novel data in part I of this thesis further support the overriding role of up-regulated wild type functioning p53 in the disease process. This assumption is supported by our novel data on MDM4, MDM4phospho, SPARC, TGFβ1 and VEGF-A expression in the epidermis of these patients.

This constant up-regulation of p53 could be based on the induction and up-regulation of $p76^{MDM2}$ and MDM4/MDM4phospho interaction in the entire epidermal compartment of these patients. This assumption is supported by the presence of $p76^{MDM2}$ splice variant which is completely absent in healthy skin.

4. Furthermore, as our results indicate the presence of up-regulated, but nitrated SPARC, we would like to suggest a novel function for this regulatory protein as possible radical scavenger in classical vitiligo based on oxidation and nitration of the protein structure. This assumption is fostered by Computer Simulation. From all results obtained, we would like to conclude that up-regulation of epidermal SPARC in classical vitiligo is directly under control by H_2O_2 which includes induction through this ROS and scavenging of ROS and RNS. This assumption is supported by prevented induction of SPARC through VEGF-A and TGFβ1, as both proteins are also affected by nitration, leading in turn to non-functional signalling with regards to SPARC regulation.

To sum up, our novel data further substantiate our original proposed idea, how patients with vitiligo are protected by a constantly up-regulated functioning p53 /p21 cascade against development of non-melanoma skin cancer and photo-damage despite absence of inherited pigment and the presence of DNA-damage. Moreover, our data suggest

that up-regulated SPARC functions as radical scavenger in classical vitiligo. This is a completely new idea.

MAL is a distinct entity compared to classical vitiligo despite the presence of epidermal oxidative / nitrative stress (PART II)

The aim of part II in this thesis was, to find out, whether melanoma associated leucoderma (MAL) is a distinct clinical entity compared to classical vitiligo. There are many reports in the literature, but at the present time, there is no common consent on this phenomenon. To approach our aim of answering the raised question, whether MAL is classical vitiligo or not, we compared both leucodermas with the same methodology under the same conditions..

1. Upon Wood's light examination we see a weak fluorescence of the white lesion in MAL, pointing to the presence of oxidised pterins. A yellowish fluorescence is the clinical hallmark for correct diagnosis of classical vitiligo (Schallreuter et al., 1994a).
2. In MAL the entire epidermis holds H_2O_2 and $ONOO^-$ accumulation proven by *in vivo* FT-Raman spectroscopy and by immuno-fluorescence. This result is the same as in classical vitiligo.
3. Based on our immuno-fluorescence data, we can conclude that MAL involves the entire epidermal compartment which is the same in classical vitiligo.

Immuno- labelled expression patterns point to fundamental differences between MAL and classical vitiligo, utilising the same protein panel including catalase, $ONOO^-$, p53, p21, $p76^{MDM2}$, MDM4, MDM4phospho, SPARC, VEGF-A and TGF-β1.

In vitiligo we always observed distribution of our protein in question throughout the entire epidermis except TGFβ1 expression.

In MAL we observed in most of the proteins a significant gradient from the basal / suprabasal to the upper layer, with higher expression in the basal / suprabasal layers, and low expression in the upper layer or in the reciprocal way. **Figures 110a, b, c summarise** these results, supporting a very different scenario compared to classical vitiligo.

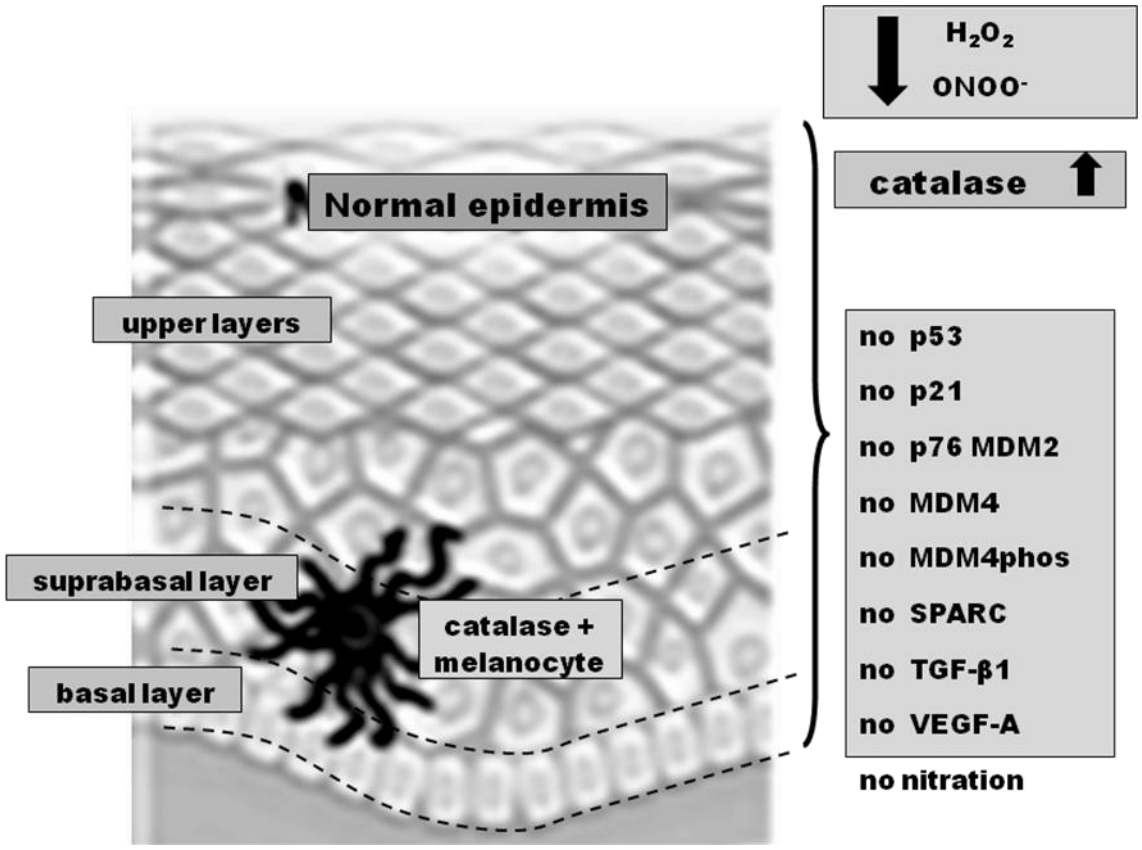

Figure 110a: Absence of all our proteins in question in healthy control epidermis but catalase is present in keratinocytes and melanocytes.

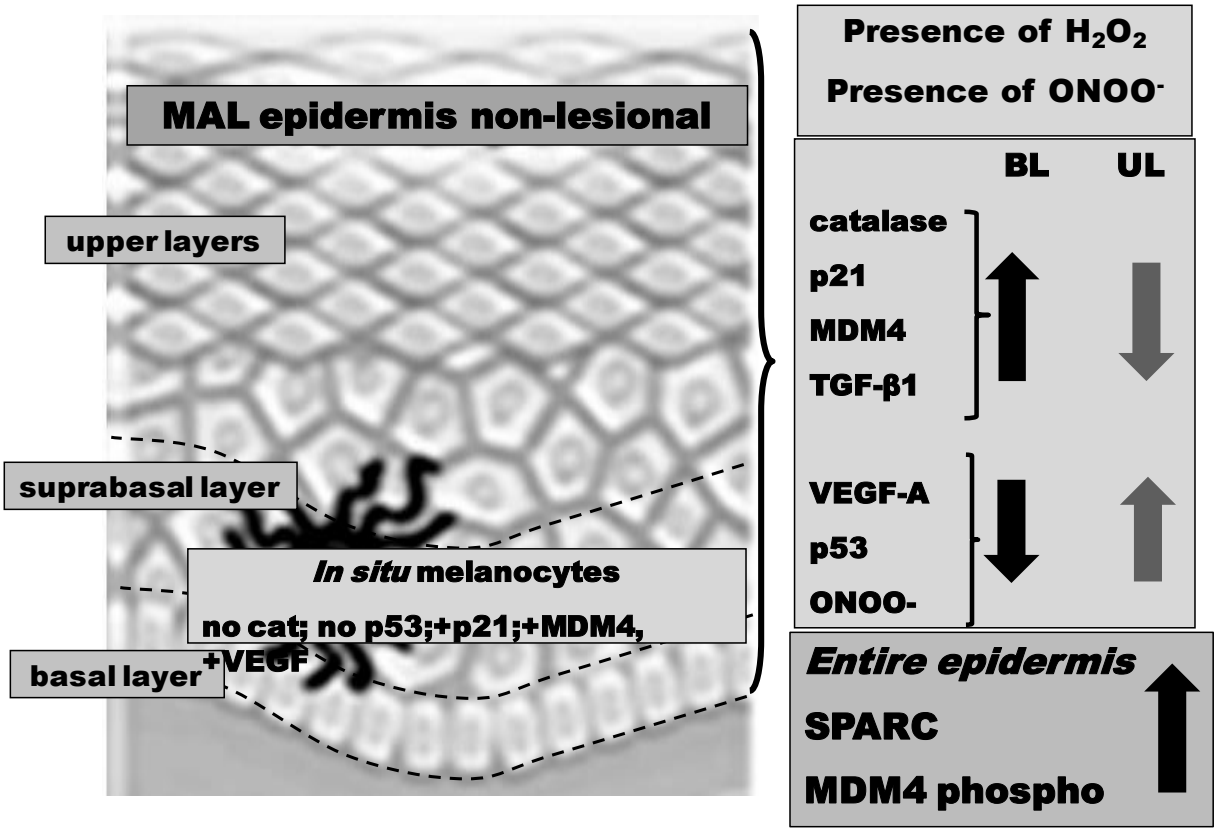

Figure 110b: Distribution of the proteins in question in MAL non-lesional epidermis.

The scheme depicts the distribution of the protein in the basal / supra basal layers (BL) and in the upper layers (UL) in the epidermis as described in detail in the result section.

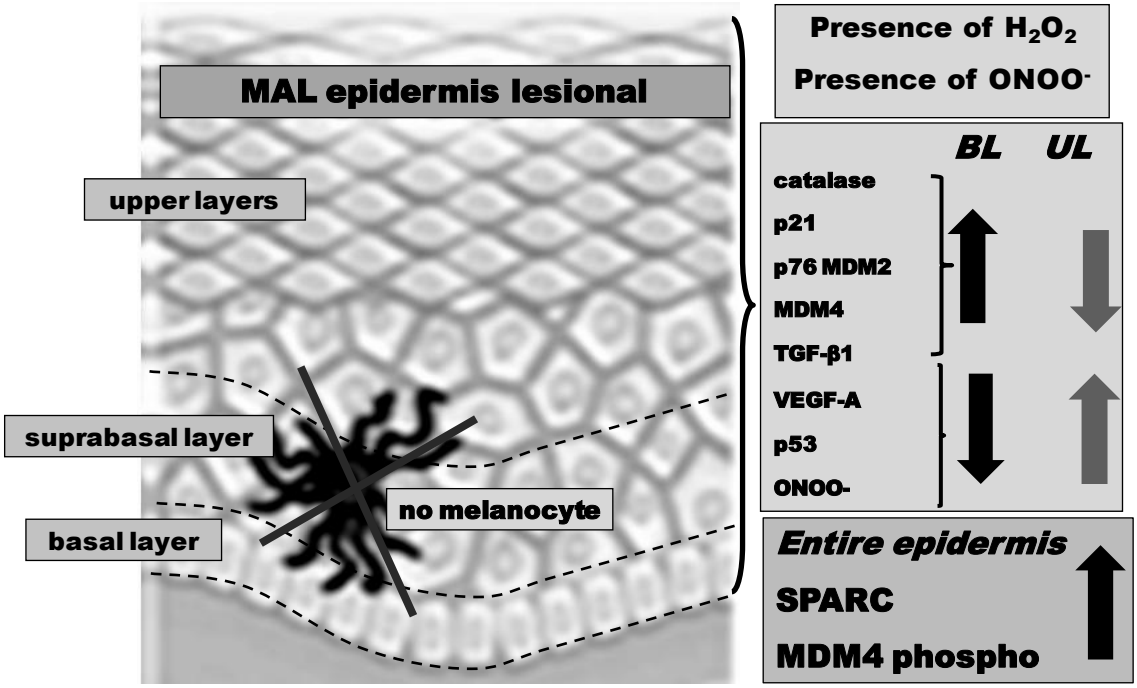

Figure 110c: Distribution of the proteins in question in MAL lesional epidermis.

The scheme depicts the distribution of the protein in the basal / supra basal layers (BL) and in the upper layers (UL) in the epidermis as described in detail in the result section.

Looking at the effect of $ONOO^-$-mediated nitration of SPARC and TGFβ1 we can see that in classical vitiligo SPARC is heavily nitrated throughout the entire epidermis which is very different in MAL. In lesional MAL SPARC is not nitrated in the basal layer with little nitration of nuclei in the upper layer. In non-lesional MAL nitrated SPARC is expressed in the nuclei of the upper layer. In the case of TGFβ1we see strong nitration in the upper layer with almost absence in the basal layer in lesional and non-lesional skin.

Based on our results it is tempting to conclude that in MAL t H_2O_2 / ONOO- accumulation ispresent but it seems to be better controlled by an effective functioning catalase. Whether other antioxidant systems including thioredoxin reductase, GPx and

glutathione reductase as well as methionine sulfoxide reductases A&B are affected in MAL needs to be shown.

The development of postsurgical depigmentation in melanoma patients is well known. Still this phenomenon is puzzling and poorly understood. In this context it is important to recognize that melanoma is a cutaneous malignancy originating from a **proliferation of atypical melanocytes**, while vitiligo is characterised by hypopigmented / depigmented skin lesions with a **loss of functional epidermal melanocytes** and aberrant funcyionality of keratinocytes (Schallreuter et al. 2008a, Tobin et al., 2000; Schallreuter, 2014; Quaglino et al., 2010; Lotti et al 2008a; b).

Based on our data we can suggest that MAL and classical vitiligo are two different entities.

Whether co-existence of MAL and classical vitiligo in patients with cutaneous melanoma could be important for tumour survival rates needs investigation of more patients. However, in this context it is interesting that our patient with MAL until 3 / 2015 had no signs of tumour progression over a period of almost 7 years since his primary surgery. Here it is also interesting that he has no other immune diseases. As the presence of those have been attributed to a favourable outcome, it seems that at least in our patient there is no connection (Quaglino et al., 2010).

12. Future work

Data presented in this thesis revealed that MAL is a distinct entity compared to classical vitiligo. The following future work may be helpful to further substantiate the differences between these two depigmentation disorders.

- Comparison of $p76^{MDM2}$ and $p90^{MDM2}$ expression in MAL.
- Identification of $p76^{MDM2}$/$p90^{MDM2}$- *in situ* levels in melanocytes of MAL non-lesional skin.
- As nitration is present in MAL, studying nitration of p21, p53, $p76^{MDM}2$, $p90^{MDM2}$, MDM4/MDM4phospho should be involved.
- Degree of melanocyte detachment in MAL compared to classical vitiligo should be an interesting topic to follow in more detail.
- Shedding more light on the possible role of TGF-β1 in promoting hypopigmentation in MAL via studying its negative effect on the expression of melanogenic proteins such as MITF, TRP-1 and TRP-2.
- Most importantly, more samples of MAL patient samples are mandatory to confirm our findings.

13. References

Abdel-Naser, M. B. (1999). Differential effects on melanocyte growth and melanization of low vs. high calcium keratinocyte-conditioned medium. *Br J Dermatol*. 140(1): 50-5.

Adams, J. D., Klaidman, Jr. L. K., Ribeiro, P. (1997). Tyrosine hydroxylase: mechanisms of oxygen radical formation. *Redox Rep*. 3(5-6): 273-9.

Aisenbrey S., Luke, C., Ayertey, H.D., Grisanti, S., Perniok, A., Brunner, R. (2003). Vogt–Koyanagi–Harada syndrome associated with cutaneous malignant melanoma: an 11-year follow-up. *Graefes Arch Clin Exp Ophthalmol*. 241: 996–999.

Akbayir, N., Gökdemir, G., Mansur, T., Sökmen, M., Gündüz, S., Alkim, C., Barutcuoglu, B., Erdem, L. (2004). Is there any relationship between hepatitis C virus and vitiligo? *J Clin Gastroenterol*. 38(9): 815-7.

Albino, A. P., Vidal, M. J., McNutt, N. S., Shea, C. R., Prieto, V. G., Nanus, D. M., Palmer, J. M,. Hayward, N. K. (1994). Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res.* 4: 35–45.

Alford, A.I., Hankenson, K.D. (2006). Matricellular proteins: Extracellular modulators of bone development, remodeling, and regeneration. *Bone*. 38,749–757.

Alikhan, A., Felsten, L. M., Daly, M., Petronic-Rosic, V. (2011). Vitiligo: a comprehensive overview Part I. Introduction, epidemiology, quality of life, diagnosis, differential diagnosis, associations, histopathology, etiology, and work-up. *J Am Acad Dermatol.* 65(3):473-91. Review.

Alkhateeb, A., Fain, P. R., Thody, A., Bennett, D. C., Spritz, R. A. (2003). Epidemiology of vitiligo and associated autoimmune diseases in Caucasian probands and their families. *Pigment Cell Res*. 16:208-14.

Al-Khawajah, M. M. (1997). Photochemotherapy for vitiligo: SEVEN years' experience at a University hospital. *Ann Saudi Med.* 17(2): 175-178.

Alon, T., Hemo, I., Itin, A., Pe'er, J., Stone, J., Keshet, E. (1995). Vascular endothlial growth factor acts as a survival factor for newly formed vessels and has implications for retinopathy of prematurity. *Nat Med.* 1:1024-1028.

Alonso, S. R., Tracey, L., Ortiz, P. Pérez-Gómez, B., Palacios, J., Pollán, M., Linares, J., Serrano, S., Sáez-Castillo, A. I., Sánchez, L., Pajares, R., Sánchez-Aguilera, A., Artiga, M. J., Piris, M. A., Rodríguez-Peralto, J. L. (2007). A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res.* 67, 3450–3460.

Alvarez, M. J., Prada, F., Salvatierra, E., Bravo, A. I., Lutzky, V. P., Carbone, C., Pitossi, F. J., Chuluyan, H. E., Podhajcer, O. L. (2005). Secreted protein acidic and rich in cysteine produced by human melanoma cells modulates polymorphonuclear leukocyte recruitment and antitumor cytotoxic capacity. *Cancer Res.* 65, 5123–5132.

Aroni, K., Voudouris, S., Ioannidis, E., Grapsa, A., Kavantzas, N., Patsouris, E. (2010). Increased angiogenesis and mast cells in the centre compared to the periphery of vitiligo lesions. *Arch Dermatol Res*. 302(8):601-7.

Aronoff, S. (1965). Catalase: kinetics of photooxidation. *Science.* 150(692): 72-3.

Arrowsmith, C. H. (1999). Structure and function in the p53 family. *Cell Death & Differentiation*. 6(12): 1169-73.

Arycan, O, Koc, K, Ersoy, L. (2008). Clinical characteristics in 113 Turkish vitiligo patients. *Acta Dermatovenerol Alp Panonica Adriat.* 17:129-32.

Avery-Kiejda, K. A., Bowden, N. A., Croft, A. J., Scurr, L. L., Kairupan, C. F., Ashton, K. A., Talseth-Palmer, B. A., Rizos, H., Zhang X. D., Scott R. J., Hersey P.(2011) . P53 in human melanoma fails to regulate target genes associated with apoptosis and the cell cycle and may contribute to proliferation. *BMC Cancer.* 11:203.

Bachmann, I. M., Straume, O., Puntervoll, H. E., Kalvenes, M. B., Akslen, L. A. (2005). Importance of P-cadherin, beta-catenin, and Wnt5a/ frizzled for progression of melanocytic tumors and prognosis in cutaneous melanoma. *Clin Cancer Res.* 11(24): 8606-8614.

Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., Rhee, S. G. (1997). Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem.* 272(1): 217-21.

Bakalkin, G., Yakovleva, T., Selivanova, G., Magnusson, K. P., Szekely, L., Kiseleva, E., Klein, G., Terenius, L., Wiman, K. G. (1994). p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc Natl Acad Sci USA*. 91(1): 413-7.

Barak, Y., Juven, T., Haffner, R., Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. *EMBO J.* 12: 461-468.

Barboza, J.A., Iwakuma, T., Terzian, T., El-Naggar, A.K., Lozano, G. (2008). Mdm2 and Mdm4 loss regulates distinct p53 activities. *Mol Cancer Res.* 6:947–54.

Bargonetti, J., Manfredi, J. J., Chen, X., Marshak, D. R., Prives, C. (1993). A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. *Genes Dev.* 7(12B): 2565-74.

Barzu, O. and Dansoreanu, M. (1980). Spectrophotometric determination of H_2O_2-generating oxidases using oxyhemoglobin as oxygen donor and indicator. *Int J Biochem.* 11(2): 121-6.

Bassuk, J. A., Pichler, R., Rothmier, J. D., Pippen, J., Gordon, K., Meek, R. L., Bradshaw, A. D., Lombardi, D., Strandjord, T. P., Reed, M., Sage, E. H., Couser, W. G., Johnson, R. (2000). Induction of TGF-beta1 by the matricellular protein SPARC in a rat model of glomerulonephritis. *Kidney Int* .57:117–128.

Bayle, J. H., Elenbaas, B., Levine, A. J. (1995). The carboxyl-terminal domain of the p53 protein regulates sequence- specific DNA binding through its nonspecific nucleic acid-binding activity. *Proc Natl Acad Sci U S A.* 92(12): 5729-33.

Behl, P. N. and Bhatia, R. K. (1971). 400 cases of vitiligo – a clinicotherapeutic analysis. *Indian J Dermatol.* 17: 51–53.

Bellahcene, A., Castronovo, V. (1995). Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. *Am. J. Pathol.* 146, 95–100.

Benjannet, S., Rondeau, N., Day, R., Chretien, M., Seidah, N. G. (1991). PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. *Proc Natl Acad Sci. USA.* 88:3564-3568.

Berking, C., Takemoto, R., Schaider, H., Showe, L., Satyamoorthy, K., Robbins, P., Herlyn, M. (2001). Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. *Cancer Res.* 61, 8306–8316.

Bhawan, J. and Bhutani L. K. (1983). Keratinocyte damage in vitiligo. *J Cutan Pathol.* 10(3): 207-12.

Blagosklonny, M. V. (1997). Loss of function and p53 protein stabilization. *Oncogene.* 1889–1893.

Blaschko, A. (1901). Die Nervenverteilung in der Haut in ihrer Beziehung zu den Erkrankungen der Haut. *Vienna and Leipzig: Braumüller.*

Boasberg, P.D., Hoon, D.S., Piro, L.D., Martin, M.A., Fujimoto, A., Kristedja, T.S., Bhachu, S., Ye, X., Deck, R.R, O'Day, S.J. (2006). Enhanced survival associated with vitiligo expression during maintenance biotherapy for metastatic melanoma. *J Invest Dermatol.* 126(12):2658-63.

Boissy, R. E. and Manga, P. (2004). On the etiology of contact/occupational vitiligo. *Pigment Cell Res*. 17(3): 208-14.

Boissy, R. E., Liu, Y. Y., Medrano, E. E., Nordlund, J. J. (1991). Structural aberration of the rough endoplasmic reticulum and melanosome compartmentalization in long-term cultures of melanocytes from vitiligo patients. *J Invest Dermatol.* 97: 395–404.

Bolognia, J. K., Orlow, S. J., Glick, S. A. (1994). Lines of Blaschko. *J Am Acad Dermatol. 31*:157-90.

Bradshaw, A. D. and Sage, E. H. (2001). SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. *J Clin Invest.* 107: 1049–54.

Brauchle, M., Funk, J. O., Kind, P., Werner, S. (1996). Ultraviolet B and H2O2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J Biol Chem.* 271: 21793–21797.

Breitbart, E. W., Greinert, R., Volkmer, B. (2006). Effectiveness of information campaigns. *Prog Biophys Mol Biol*. 92: 167-172.

Brekken R.A. and Sage, E.H. (2000). SPARC, a matricellular protein: At the crossroads of cell matrix. *Matrix Biol.* 19,569–580.

Brekken, R. A. and Sage, E. H. (2001). SPARC, a matricellular protein: at the crossroads of cell-matrix communication. *Matrix Biol. 19*: *816*–27.

Brenner M. and Hearing V.J. (2008). The protective role of melanin against UV damage in human skin. *Photochem Photobiol.* 84(3):539-49.

Brown, L. F., Yeo, K.T., Berse, B., Yeo, T.K., Senger, D R., Dvorak, H.F., van de Water, L. (1992). Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med*. 176: 1375–1379.

Burney, S., Caulfield, J.L., Niles, J.C., Wishnok, J.S., Tannenbaum, S.R. (1999). The chemistry of DNA damage from nitric oxide and peroxynitrite. *Mutat Res.* 424(1-2): 37-49.

Burns, T., Breathnach, S., Cox, N., Griffiths C. (2004). Rook`s textbook of dermatology. Volume II. Seventh edition. *Blackwell Publishing Company.*

Bystryn, J.C., Rigel, D., Friedman, R.J., Kopf, A. (1987). Prognostic significance of hypopigmentation in malignant melanoma. *Arch Dermatol*. .123(8):1053-5.

Cahilly-Snyder, L., Yang-Feng T., Francke, U., George, D.L. (1987). Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somat. Cell. Mol. Genet.* 13, 235-244.

Calanchini-Postizzi, E. and Frenk, E. (1987). Long-term actinic damage in sun-exposed vitiligo and normally pigmented skin. *Dermatologica*. 174(6): 266- 271.

Candeias, M.M., Malbert-Colas, L., Powell, D.J., Daskalogianni, C., Maslon, M.M., Naski, N., Bourougaa, K., Calvo, F., Fåhraeus, R. (2008). P53 mRNA controls p53 activity by managing Mdm2 functions. *Nat Cell Biol.* 10:1098-105.

Carter, S., Bischof, O., Dejean, A., Vousden, K.H. (2007). C-terminal modifications regulate MDM2 dissociation and nuclear export of p53. Nat Cell Biol. 9: 428-435.

Casp, C. B., She, J.X., McCormack, W.T. (2002). Genetic association of the catalase gene (CAT) with vitiligo susceptibility. *Pigment Cell Res.* 15(1): 62-6.

Cerdan, D., Redziniak, G., Bourgeois, C. A., Monsigny, M., Kieda, C. (1992). C32 human melanoma cell endogenous lectins: characterization and implication in vesicle-mediated melanin transfer to keratinocytes. *Exp Cell Res.* 203: 164–173.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., Bernstein, A. (1988). The proto-oncogene c-Kit encoding the transmembrane tyrosine kinase receptor maps to the mouse W locous. *Nature.* 335, 88-89.

Chandrashekar, L. (2009). Dermatoscopy of blue vitiligo. *Clin Exp Dermatol*. 34:e125-6.

Chavan, B., Gillbro J. M., Rokos, H., Schallreuter, K. U. (2006). GTP cyclohydrolase feedback regulatory protein controls cofactor 6-tetrahydrobiopterin synthesis in the cytosol and in the nucleus of epidermal keratinocytes and melanocytes. *J Invest Dermatol.* 126(11): 2481-9.

Chedekel, M.R. (1982). Photochemistry and photobiology of epidermal melanins. *Photochem Photobiol.* 35:881–885.

Chedekel, M.R., Post, P.W., Deibel, R.M., Kalus, M. (1977). Photodestruction of phaeomelanin. *Photochem Photobiol.* 26:651–653.

Chedekel, M.R., Smith, S.K., Post, P.W., Pokora, A., Vessell, D.L. (1978). Photodestruction of pheomelanin: role of oxygen. *Proc Natl Acad Sci USA.* 75:5395–5399.

Cheetham, S., Tang, M.J., Mesak, F., Kennecke, H., Owen, D., Tai, I.T. (2008). SPARC promoter hypermethylation in colorectal cancers can be reversed by 5-Aza-2_deoxycytidine to increase SPARC expression and improve therapy response. *Br. J. Cancer.* 98, 1810–1819.

Chehab, N.H., Malikzay, A., Stavridi, E.S., Halazonetis, T.D. (1999). Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proceedings of the *National Academy of Sciences of the United States of America. 96*, 13777-13782.

Cheli, Y., Ohanna, M., Ballotti, R., Bertolotto, C. (2009). Fifteen-year quest for microphthalmiaassociated transcription factor target genes. *Pigment Cell Melanoma Res.* 23:27–40.

Chen, J., Marechal, V., Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. *Mol. Cell.Biol.* 13, 4107–4114.

Chen, L., Gilkes, D.M., Pan, Y., Lan,e W.S., Chen, J. (2005b). ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *EMBO J.* 24: 3411–22.

Chen, L., Li, C., Pan, Y., Chen, J. (2005a). Regulation of p53-MDMX interaction by casein kinase 1 alpha. *Mol Cell Biol.* 25, 6509-6520.

Cheng, T.-H., Cohen, S.N. (2007). Human MDM2 isoforms translated differentially on constitutive versus p53-regulated transcripts have distinct functions in the p53/MDM2 and TSG101/MDM2 feedback control loops. *Mol Cell Biol.* 27: 111-119.

Chiaverini, C., Beuret, L., Flori, E., Busca, R., Abbe, P., Bille, K., Bahadoran, P., Ortonne, J. P., Bertolotto, C., Ballotti, R. (2008). Microphthalmia-associated transcription factor regulates RAB27A gene expression and controls melanosome transport. *J. Biol. Chem.* 283: 12635-12642.

Chlenski A., Liu, S.Q., Bake,r L.J., Yang ,Q.W., Tian, Y.F., Salwen, H.R., Cohn, S.L. (2004). Neuroblastoma angiogenesis is inhibited with a folded synthetic molecule corresponding to the epidermal growth factorβ like module of the follistatin domain of SPARC. *Cancer Res.* 64: 7420–7425.

Chlenski, A., Cohn, S. L. (2010). Modulation of matrix remodeling by SPARC in neoplastic progression. *Semin Cell Dev Biol.* 21: 55–65.

Cho, Y., Gorina, S., Jeffrey, P. D., Pavletich, N. P. (1994). Crystal structure of a p53 tumor suppressor- DNA complex: understanding tumorigenic mutations. *Science.* 265(5170): 346-55.

Christiansen, J. H., Coles, E. G., Wilkinson, D. G. (2000). Molecular control of neural crest formation, migration and differentiation. *Curr Opin Cell Biol.* 12 (6): 719–24.

Christophers, S. and Braver, S. (1987). *Elementa Dermatologica.*

Claffey, K.P., Brown, L.F., del Aguila, L.F., Tognazzi, K., Yeo K.T., Manseau, E.J., Dvorak, H.F. (1996). Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. *Cancer Res.* 56: 172–181.

Clague, M.J. (2002). Membrane transport: a coat for ubiquitin. *Curr Biol.* 12(15):R529-31.

Cobbs, C.S., Brenman, J.E., Aldape, K.D., Bredt, D.S., Israel, M.A. (1995). Expression of nitric oxide synthase in human central nervous system tumors. *Cancer Res.* 55(4): 727-30.

Cobbs, C.S., Samanta, M., Harkins, L.E., Gillespie, G.Y., Merrick, B.A., MacMillan-Crow, L.A. (2001). Evidence for peroxynitrite-mediated modifications to p53 in human gliomas: possible functional consequences. *Arch Biochem Biophys.* 394(2): 167-72.

Cruickshank, C.N. and Harcourt, S.A. (1964). Pigment donation in vitro. *J Invest Dermatol.* 42: 183–184.

Cucchi, M. L., Frattini, P., Santagostino, G., Preda, S., Orecchia, G. (2003). Catecholamines increase in the urine of non-segmental vitiligo especially during its active phase. *Pigment Cell Res.* 16:111–116.

Cui, R., Widlund, H.R., Feige, E., Lin, J.Y., Wilensky, D.L., Igras, V.E., D'Orazio, J., Fung, C.Y., Schanbacher, C.F., Granter, S.R., Fisher, D.E.(2007). Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell.* 128: 853–864.

Cunha, D., Pacheco, F.A., Cardoso, J. (2009). Vitiligo: A good prognostic factor in melanoma? *Dermatol. Online J.* 15 (2): 15.

Damjanovski, S., Huynh, M.H., Motamed, K., Sage, E.H., Ringuette, M., (1998). Regulation of SPARC expression during early *Xenopus* development: evolutionary divergence and conservation of DNA regulatory elements between amphibians and mammals. *Dev. Genes Evol.* 207: 453-461.

Daneshpazhooh, M.,Mostofizadeh, G. M., Behjati, J., Akhyani, M., Mahmoud Robati, R. (2006). Anti-thyroid peroxidise antibody and vitiligo: a controlled study. *BMC Dermatol.* 6:3.

Daniotti, M., Oggionni, M., Ranzani, T., Vallacchi, V., Campi, V., Di Stasi, D., Torre, G. D., Perrone, F., Luoni, C., Suardi, S., Frattini, M., Pilotti, S., Anichini, A., Tragni, G., Parmiani, G., Pierotti, M. A., Rodolfo, M.(2004). BRAF alterations are associated with complex mutational profiles in malignant melanoma. *Oncogene.* 23: 5968–5977.

Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., Helin, K., Pelicci, P.G., Jochemsen, A.G., Marine, J.C. (2004). Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol.* 24(13):5835-43.

Darr, D. and Fridovich, I. (1994). Free radicals in cutaneous biology. *J Invest Dermatol.* 102: 671–675.

Das, S. K., Majumder, P. P., Chakraborty, R., Majumdar, T. K., Haldar, B. (1985). Studies on vitiligo. I. Epidemiological profile in Calcutta, India. *Genet Epidemiol.* 2(1): 71-8.

Datto, M.B., Li,Y., Panus, J.F., Howe, D.J., Xiong, Y., Wang, X.F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc. Natl. Acad. Sci. USA.* 92 5545.

Davies, R.J.H. (1996). Ultraviolet radiation damage in DNA. *Sci Am.* 275:38-43.

Debacq-Chainiaux, F., Borlon, C., Pascal, T., Royer, V., Eliaers, F., Ninane, N., Carrard, G., Friguet, B., de Longueville, F., Boffe, S., Remacle, J., Toussaint, O. (2005). Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-beta1 signaling pathway. *J Cell Sci.* 15; 118(Pt 4):743-58.

Derynck, R. and Feng, X.H. (1997). TGF-beta receptor signaling. *Biochim. Biophys. Acta.* 1333(2):F105-50J.

Detmar, M., Brown, L.F., Claffey, K.P., Yeo, K.T., Kocher, O., Jackman, R.W., Berse, B., Dvorak, H.F. (1994). Overexpression of vascular permeability factor/vascular endothelial growth factor and its receptors in psoriasis. *J Exp Med* .180: 1141–1146.

Detmar, M., Yeo, K.T., Nagy, J.A., Van de Water, L., Brown, L.F., Berse, B., Elicker, B.M., Ledbetter, S., Dvorak, H.F. (1995). Keratinocytederived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. *J Invest Dermatol.* 105: 44–50.

Di Pietro, S. M., Falcón-Pérez, J. M., Tenza, D., Setty, S. R., Marks, M. S., Raposo, G. and Dell-Angelica, E. C. (2006). BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol. Biol. Cell.* 17: 4027-4038.

Diepgen, T.L. and Mahler, V. (2002). The epidemiology of skin cancer. *Br J Dermatol.* 62: 1-6.

DiMartino, J.F., Lacayo, N.J., Varadi, M., Li, L., Saraiya, C., Ravindranath, Y., Yu, R., Sikic, B.I., Raimondi, S.C., Dahl, G.V. (2006). Low or absent SPARCexpression in acute myeloid leukemia withMLLrearrangements is associated with sensitivity to growth inhibition by exogenous SPARC protein. *Leukemia.* 20: 426–432.

Dogra, S., Parsad D., Handa S., J. Kanwar A. (2005). Late onset vitiligo: a study of 182 patients. *Int J Dermatol.* 44(3): 193-6.

Dutta, A., Ruppert, J. M., Aster, J. C., Winchester, E. (1993). Inhibition of DNA replication factor RPA by p53. *Nature.* 365(6441): 79-82.

Dvorak, H. F., Brown, L. F., Detmar, M., Dvorak, A. M. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeabilty, and angiogenesis. *Am J Pathol.* 146: 1029-1039.

Eipper, B.A. and Mains, R.E. (1980). Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocr Rev.* 1(1):1-27.

El Mofty, A. M. (1968). Vitiligo and Psoralens. *New York, Pergamon Press.*

El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell.* 75(4): 817-25.

El-Din Anbar, T., Abdel-Rahman, A.T., El-Khayyat, M.A., El-Azhary, A.E. (2008).Vitiligo on anterior aspect of neck in Muslim females: case series. *Int J Dermatol.* 47:178-9.

Elenbaas, B., Dobbelstein, M., Roth, J., Shenk, T., Levine, A.J. (1996). The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol Med.* 2:439-51.

Elias, P. M. and Feingold, K. R. (1992). Lipids and the epidermal water barrier: metabolism, regulation, and pathophysiology. *Semin Dermatol.* 11(2): 176-82.

Elwary, S. M., Chavan, B., Schallreuter, K.U. (2006). The vesicular acetylcholine transporter is present in melanocytes and keratinocytes in the human epidermis. *J Invest Dermatol.* 126(8): 1879-84.

Embden, G. and Baldes K. (1913). Űber den Abbau des Phenylalanins im tierischen Organismus. *Biochem. Z.* 55: 301-22.

Erden Inal, M., Kahraman, A., Köken, T. (2001). Beneficial effects of quercetin on oxidative stress induced by ultraviolet *A*. *Clin Exp Dermatol.* 26: 536–9.

Ezzedine, K, Lim, H.W., Suzuki, T., Katayama, I., Hamzavi, I., Lan, C.C., Goh, B.K., Anbar, T., Silva de Castro, C., Lee, A.Y., Parsad, D., van Geel, N., Le Poole, I.C., Oiso, N., Benzekri, L., Spritz, R., Gauthier, Y., Hann, S.K., Picardo, M., Taieb, A. (2012). Revised classification/nomenclature of vitiligo and related issues: the Vitiligo Global Issues Consensus Conference. *Pigment Cell Melanoma Res.* (3):E1-13

Fang, S., Jensen, J.P., Ludwig, R.L., Vousden, K.H., Weissman, A.M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem.* 275: 8945-8951.

Farrokhi, S., Hojjat-Farsangi, M., Noohpisheh, M.K., Tahmasbi, R., Rezaei, N. (2005). Assessment of the immune system in 55 Iranian patients with vitiligo. *J Eur Acad Dermatol Venereol.* 19(6): 706-11.

Feingold, K. R. (2007). Thematic review series: skin lipids. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J Lipid Res.* 48(12): 2531-46.

Feingold, K.R., Man, M.Q., Menon, G.K., Cho, S.S., Brown, B.E., Elias, P.M. (1990). Cholesterol synthesis is required for cutaneous barrier function in mice. *J Clin Invest.* 86(5): 1738-45.

Felix, C.C., Hyde, J.S., Sarna, T., Sealy, R.C. (1978). Melanin photoreactions in aerated media: electron spin resonance evidence for production of superoxide and hydrogen peroxide. *Biochem Biophys Res Commun*. 84:335–341.

Fenouille, N., Puissant, A., Tichet, M., Zimniak, G., Abbe, P., Mallavialle, A., Rocchi, S., Ortonne, J.P., Deckert, M., Ballotti, R., Tartare-Deckert, S. (2011b). SPARC functions as anti-stress factor by inactivating p53

through AKT-mediated MDM2 phosphorylation to promote melanoma cell survival. *Oncogen.* 30: 4887-4900.

Fenouille, N., Robert, G., Tichet, M., Puissant, A., Dufies, M., Rocchi, S., Ortonne, J.P., Deckert, M., Ballotti, R., Tartare-Deckert, S. (2011a). The p53/p21Cip1/ Waf1 pathway mediates the effects of SPARC on melanoma cell cycle progression. *Pigment Cell Melanoma Res.* 24 (1):219-32.

Fitzpatrick, T. B. (1964). Hypomelanosis. *South Med J.* 57: 995-1005.

Fitzpatrick, T.B. and Breathnach, A.S. (1963). The Epidermal Melanin Unit System. *Dermatol Wochenschr.*147: 481-9.

Fitzpatrick, T.B. and Mihm, M. C.J.(1971). Abrormalities in the melanin pigmentary system, in dermatology in general medicine. Edited by T. B. Fitzpatrick et al. *New York, McGraw-Hill.* pp; 1591-1637.

Fitzpatrick, T.B., Miyamoto, M., Ishikawa, K. (1967). The evolution of concepts of melanin biology. *Arch Dermatol.* 96(3): 305-23.

Fleischman, R. A., Saltman, D. L., Stastny, V., Zneimer, S. (1991). Deletion of the c-kit protooncogene in the human developmental defect piebald trait. *Proc Natl Acad Sci USA.* 88 (23): 10885–9.

Francki, A., McClure, T. D., Brekken, R. A., Motamed, K., Murri, C., Wang, T., Sage, E. H. (2004). SPARC regulates TGF-beta1-dependent signaling in primary glomerular mesangial cells. *J Cell Biochem.* 91:915–925.

Frank, S., Stallmeyer, B., Kämpfer, H., Kolb, N., Pfeilschifter, J.(1999). Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair. *FASEB J.* 13(14):2002-14.

Frippiat, C., Chen, Q.M., Zdanov, S., Magalhaes, J.P., Remacle, J., Toussaint, O. (2001). Subcytotoxic H2O2 stress triggers a release of transforming growth factor-beta 1, which induces biomarkers of cellular senescence of human diploid fibroblasts. J Biol Chem. 276(4):2531-7.

Gajjar, M., Candeias, M.M., Malbert-Colas, L., Mazars, A., Fujita, J., Olivares-Illana, V., Fåhraeus, R. (2012). The p53 mRNA-Mdm2 interaction controls Mdm2 nuclear trafficking and is required forp53 activation following DNA damage. *Cancer Cell.* 21(1):25-35.

Galarza, C., Ronceros G., Ramos W., Ortega, A., Oré, R., Ávila, J., Chía, H., Meléndez, R., Ráez, E., Hurtado, J., Escalante, M., León, I., Lindo, G., Hancco, J., Gámez, D. (2004). Cytomegalovirus skin infection in relation with the initial pathogenic mechanism of early generalized Vitiligo. *Dermatología Perlana.* 14 (3).

Gawkrodger, D.J., Ormerod, A. D., Shaw, L., Mauri-Sole, I., Whitton, M.E., Watts, M. J., Anstey, A.V., Ingham, J., Young, K. (2008). Guideline for the diagnosis and management of vitiligo. *Br J Dermatol.* 159:1051-76.

Gembarska, A., Luciani, F., Fedele, C., Russell, E.A., Dewaele, M., Villar, S., Zwolinska, A., Haupt, S., de Lange, J., Yip, D., Goydos, J., Haigh, J.J., Haupt, Y., Larue, L., Jochemsen, A., Shi, H., Moriceau, G., Lo, RS., Ghanem, G., Shackleton, M., Bernal, F., Marine, J.C. (2012). MDM4 is a key therapeutic target in cutaneous melanoma. *Nat Med.* 18(8):1239-47

Giaccia, A.J. and Kastan, M.B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* 12(19): 2973-83.

Gibbons, N. C., Wood, J. M., Rokos, H., Schallreuter, K. U. (2006).Computer simulation of native epidermal enzyme structures in the presence and absence of hydrogen peroxide (H_2O_2): potential and pitfalls. *J Invest Dermatol.* 126: 2576–2582.

Giglio, S., Mancini, F., Pellegrino, M., Di Conza, G., Puxeddu, E., Sacchi, A., Pontecorvi, A., Moretti, F. (2010). Regulation of MDM4 (MDMX) function by p76MDM2: a new facet in the control of p53 activity. *Oncogene.* 4: 29(44):5935-45.

Gillbro, J. M., L. K. Marles, Hibberts, N. A., Schallreuter, K. U. (2004). Autocrine catecholamine biosynthesis and the beta-adrenoceptor signal promote pigmentation in human epidermal melanocytes. *J Invest Dermatol.* 123(2): 346-53.

Gille, J., Reisinger, K., Asbe-Vollkopf, A., Hardt-Weinelt, K., Kaufmann, R. (2000). Ultraviolet-A-induced transactivation of the vascular endothelial growth factor gene in HaCaT keratinocytes is conveyed by activator protein- 2 transcription factor. *J Invest Dermatol.* 115: 30–36.

Glassman, S.T. (2011). Vitiligo, reactive oxygen species and T-cells. *Clinical Science.* 120: 99–120.

Gogas, H., Ioannovich, J., Dafni, U., Stavropoulou-Giokas, C., Frangia, K., Tsoutsos, D., Panagiotou, P., Polyzos, A., Papadopoulos, O., Stratigos, A., Markopoulos, C., Bafaloukos, D., Pectasides, D., Fountzilas, G., Kirkwood, J.M. (2006). Prognostic significance of autoimmunity during treatment of melanoma with interferon.*N Engl J Med.* 354(7):709-18.

Graells, J., Vinyals, A., Figueras, A., Llorens, A., Moreno, A., Marcoval, J., Gonzalez, F.J., Fabra, A. (2004). Overproduction of VEGF concomitantly expressed with its receptors promotes growth and survival of melanoma cells through MAPK and PI3K signaling. *J Invest Dermatol.*123 (6):1151-1161.

Graham-Brown, R. and Bourke, J.F. (1998). Mosby`s color atlas and text of dermatology. *Mosby international limited.*

Graham-Brown, R. and Burns, T. (2011). Dermatology. Lecture notes. 10th edition. *A John Wiley and sons, Ltd.*

Graham, A., Westerhof, W. Thody, A.J. (1999). The expression of alpha-MSH by melanocytes is reduced in vitiligo. *Ann. N. Y. Acad. Sci.* 885, 470–473.

Grimes, P.E., Sevall, J.S., Vojdani, A. (1996). Cytomegalovirus DNA identified in skin biopsy specimens of patients with vitiligo. *J Am Acad Dermatol.* 35(1): 21- 6.

Grimes, P.E, Elkadi, T., Sanders, J. (1999). Epstein-Barr virus infection in patients with vitiligo (abstr). *J Invest Dermatol.* 112: 604.

Grimes, P.E. and Billips, M. (2000). Childhood vitiligo: clinical spectrum and therapeutic approaches. In: Hann SK, Nordlund JJ, eds. Vitiligo: A Monograph on Basic and Clinical Science. *Oxford: Blackwell Scientific.* 61–9.

Groves, J.T. (1999). Peroxynitrite: reactive, invasive and enigmatic. *Curr Opin Chem Biol.* 3: 226–235.

Gu, W., and Roeder, R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell.* 90: 595-606.

Gudas, J. M., Nguyen, H., Klein, R.C., Katayose, D., Seth, P., Cowan, K.H. (1995). Differential expression of multiple MDM2 messenger RNAs and proteins in normal and tumorigenic breast epithelial cells. *Clin Cancer Res.* 1(1): 71-80.

Gudbjartsson, D.F., Sulem, P., Stacey, S.N., Goldstein, A.M., Rafnar, T., Sigurgeirsson, B., Benediktsdottir, K.R., Thorisdottir, K., Ragnarsson, R., Sveinsdottir, S.G., Magnusson, V., Lindblom, A., Kostulas, K., Botella-Estrada, R., Soriano, V., Juberías, P., Grasa, M., Saez, B., Andres, R., Scherer, D., Rudnai, P., Gurzau, E., Koppova, K., Kiemeney, L.A., Jakobsdottir, M., Steinberg, S., Helgason, A., Gretarsdottir, S., Tucker, M.A., Mayordomo, J.I., Nagore, E., Kumar, R., Hansson, J., Olafsson, J.H., Gulcher, J., Kong, A., Thorsteinsdottir, U., Stefansson, K. (2008). *ASIP* and *TYR* pigmentation variants associate with cutaneous melanoma and basal cell carcinoma. *Nat Genet.* 40:886–891.

Gumienny, T.L. and Padgett, R.W. (2002). The other side of TGFbeta superfamily signal regulation: Thinking outside the cell. Trends in Endocrinological Metabolism. 13: 295–299.

Gwosdz, C., Scheckenbach, K., Lieven, O., Reifenberger, J., Knopf, A., Bier, H., Balz, V. (2006). Comprehensive analysis of the p53 status in mucosal and cutaneous melanomas. *Int J Cancer*. 118:577-582.

Haak, A., Scott, G.A., Holbrook, K. (2001). In The biology of the skin. Freinkel, R.K., Woodley, D.T. (2001). Chapter 2. *The parthenon publishing group.*

Haass, N.K., Smalley, K.S., Herlyn, M. (2004). The role of altered cell–cell communication in melanoma progression. *J. Mol. Histol.* 35, 309–318.

Haass, N.K. and Herlyn, M. (2005). Normal human melanocytes homostasis as a paradigm for understanding melanoma. *J Invest Dermatol Symp Proc.* 10: 153-163.

Haass, N.K., Smalley, K.S., Li, L., Herlyn, M. *(*2005b*)*. Adhesion, migration and communication in melanocytes and melanoma. *Pigment Cell Res.* 18, 150–159.

Haines, D. S., Landers, J. E., Engle, L.J., George, D.L. (1994). Physical and functional interaction between wild-type p53 and mdm2 proteins. *Mol Cell Biol.* 14(2): 1171-8.

Halazonetis, T.D., Davis, L.J., Kandil, A.N. (1993). Wild-type p53 adopts a mutant-like conformation when bound to DNA. *Embo J.* 12(3): 1021-8.

Halder, R.M., and Nootheti, P.K. (2003). Ethnic skin disorders overview. *J Am Acad Dermatol.* 48(6S): 143–148.

Halder, R.M. and Taliaferro, S.J. (2008). Vitiligo. In: Wolff, K., Goldsmith, L., Katz, S., Gilchrest, B., Paller, A., Lefell, D., editors. Fitzpatrick's dermatology in general medicine. *New York: McGraw-Hill.* p. 72.

Hall, C.J. (2000). Sauer`s manual of skin diseases. Eighth edition. *Lippincott Williams &Wilkins. USA.* Chapter one.

Hammer, J.A. and Wu, X.S. (2002). Rabs grab motors: defining the connections between Rab GTPases and motor proteins. *Curr. Opin. Cell Biol.* 14: 69-75.

Handa, S. and Kaur, I. (1999). Vitiligo: clinical findings in 1436 patients. *J Dermatol.* 26(10): 653-7.

Hann, S.K. (2000). Clinical features of segmental vitiligo. In: Hann SK, Nordlund JJ, eds. Vitiligo: A Monograph on the Basic and Clinical Science. *Oxford: Blackwell Scientific. 49*–60.

Hann, S.K. and Lee, H.J. (1996). Segmental vitiligo: clinical findings in 208 patients. *J Am Acad Dermatol.* 35(5 Pt 1): 671-4.

Hann, S.K., Koo, S. W., Kim, J.B., Park, Y.K. (1996). Detection of antibodies to human melanoma cells in vitiligo and alopecia areata by Western blot analysis. *J Dermatol.* 23(2):100–3.

Hann, S.K. and Chun, W. (2000). Autocytotoxic hypothesis for the destruction of melanocytes as the cause of vitiligo. In: Hann, S. K., Nordlund, J., editors. Vitiligo. *Oxford: Blackwell Science Ltd.*

Hann, S.K., Kim, Y.S., Yoo, J.H., Chun, Y. S. (2000). Clinical and histopathologic characteristics of trichrome vitiligo. *J Am Acad Dermatol.* 42:589-96.

Hann, S.K. and Im, S. (2004). Clinical variants of Vitiligo. Vitiligo: Problems and Solutions. T. H. Lotti. *New York and Basel, Marcel Dekker, Inc.* 29: 159-172.

Happle, R. (1977). Genetische Bedeutung der Blaschkoschen Linien. *Z Hautkr.* 52:935–944.

Harper, D.C., Theos, A.C., Herman, K.E., Tenza, D., Raposo, G., Marks, M.S. (2008). Premelanosome amyloid-like fibrils are composed of only golgi-processed forms of Pmel17 that have been proteolytically processed in endosomes. *J. Biol. Chem.* 283: 2307-2322.

Harris, C.C. (1993). p53: at the crossroads of molecular carcinogenesis and risk assessment. *Science.* 262(5142): 1980-1.

Harris, R.B., Griffith, K., Moon, T.E. (2001). Trends in the incidence of nonmelanoma skin cancers in southeastern Arizona, 1985–1996. *J Am Acad Dermatol.* 45(4):528 –536.

Hartmann, A., Bedenk, C., Keikavoussi, P., Becker, J.C., Hamm, H., Bröcker, E.B. (2008).Vitiligo and melanoma-associated hypopigmentation (MAH): shared and discriminative features. *J Dtsch Dermatol Ges.*6 (12):1053-9.

Hartwell, L. (1992). Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell.* 71(4): 543-6.

Hartwell, L.H. and Kastan, M.B. (1994). Cell cycle control and cancer. *Science.* 266(5192): 1821-8.

Hasse, S., Kothari, S., Rokos, H., Kauser, S., Schürer, N.Y., Schallreuter, K.U.(2005). *In vivo* and *in vitro* evidence for autocrine DCoH/HNF-1alpha transcription of albumin in the human epidermis. *Exp Dermatol.* 14(3): 182-7.

Hasselaar, P. and Sage, E.H (1992) SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. *J Cell Biochem.* 49:272–283.

Haupt, Y., Maya, R., Kazaz, A., Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature.* 387(6630):296-9.

He, Z., Brinton, B.T., Greenblatt, J., Hassell, J.A., Ingles, C.J. (1993). The transactivator proteins VP16 and GAL4 bind replication factor A. *Cell.* 73(6): 1223-32.

Hearing, V. J. (2005). Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J. Dermatol. Sci.* 37: 3-14.

Heck, D.E., Gerecke, D.R., Vetrano, A.M., Laskin, J.D. (2004). Solar ultraviolet radiation as a trigger of cell signal transduction. *Toxicol Appl Pharmaco.* 195: 288–297.

Henle, F.G.J. (1837). Symbolae ad anatomiam villorum intestinalum, imprismis eorum epithelii et vasorum lactorum. Berlin.

Hodi, F.S., O'Day, S.J., McDermott, D.F., Weber, R.W., Sosman, J.A., Haanen, J.B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J.C., Akerley, W., van den Eertwegh, A.J., Lutzky, J., Lorigan, P., Vaubel, J.M., Linette, G.P., Hogg, D., Ottensmeier, C.H., Lebbé, C., Peschel, C., Quirt, I., Clark, J.I., Wolchok, J.D., Weber, J.S., Tian, J., Yellin, M. J., Nichol, G.M., Hoos, A., Urba, W.J. (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* 363:711–723.

Hoek, K.S., Schlegel, N.C., Brafford, P., Sucker, A., Ugurel S., Kumar, R., Weber, B.L., Nathanson, K.L., Phillips, D.J., Herlyn, M., Schadendorf, D., Dummer, R. (2006). Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. *Pigment Cell Res.* 19: 290– 302.

Hoffman, M. and Weinberg, J.B. (1987). Tumor necrosis factor-alpha induces increased hydrogen peroxide production and Fc receptor expression, but not increased Ia antigen expression by peri, toneal macrophages. *J Leukoc Biol.* 42(6): 704-7.

Hohenester, E., Maurer, P., Hahoenadl, C., Timpl, R., Jansonius, J.N., Engel, J. (1996). Structure of a novel extracellular Ca(2+)-binding module in BM-40. *Nat Struct. Biol.* 3:67-73.

Hohenester, E., Maurer, P., Timpl, R. (1997). Crystal structure of a pair of follistatinlike and EF-hand calcium-binding domains in BM-40. *EMBO J.* 16: 3778–3786.

Hohenester, E., Sasaki, T., Giudici, C., Farndale, R.W., Bachinger, H.P. (2008). Structural basis of sequence-specific collagen recognition by SPARC. *Proc Natl Acad Sci USA.* 105:18273–18277.

Hohenester, E., Maurer P., Timpl R. (1997). Crystal structure of a pair of follistatin-like and EF-hand calcium-binding domains in BM-40. *EMBO J.* 16 (13): 3778–3786.

Holbrook, K.A. and Wolff, K. (1993). The structure and development of skin. Dermatology in General Medicine. Fitzpatrick, P. F., Eisen, A. Z., Wolff, K., Freedberg, I. M., Austen, K. F., *McGraw Hill.* 1: 97-145.

Hollbrook, K.A. (1998). Melanocytes in human embryonic skin and fetal skin: review and new findings. *Pigment Cell Res.* 1(Suppl): 6–17.

Hollstein, M., Sidransky, D., Vogelstein, B, Harris, C.C. (1991). p53 mutations in human cancers. *Science.* 253(5015): 49-53.

Honda, R. and Yasuda, H. (2000). Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. *Oncogene.* 19: 1473-1476.

Honda, R., Tanaka, H., Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* 420: 25-27.

Houben, R., Hesbacher, S., Schmid, C.P., Kauczok, C.S., Flohr, U., Haferkamp, S., Müller, C.S., Schrama, D., Wischhusen, J., Becker, J.C. (2011). High-level expression of wild-type p53 in melanoma cells is frequently associated with inactivity in p53 reporter gene assays. *PLoS One.* 6(7):e22096.

Huizing, M., Sarangarajan, R., Strovel, E., Zhao, Y., Gahl, W.A. and Boissy, R.E. (2001). AP-3 mediates tyrosinase but not TRP-1 trafficking in human melanocytes. *Mol. Biol. Cell.*12: 2075-2085.

Hussein, M.R. (2005). Ultraviolet radiation and skin cancer: molecular mechanisms. J Cutan Pathol. 32: 191-205.

Hyytiainen, M., Penttinen, C., Keski-Oja, J. (2004). Latent TGF-β binding proteins: extracellular matrix association and roles in TGF-β activation. *Crit Rev Clin Lab Sci.* 41: 233-264.

Imokawa, G., and Mishima, Y. (1986). Importance of glycoproteins in the initiation of melanogenesis: an electron microscopic study of B-16 melanoma cells after release from inhibition of glycosylation. *J Invest Dermatol.* 87(3):319-25.

Ischihashi, M., Ueda, M., Budiyanto, A., Bito T., Oka, M., Fukunaga, M., Tsuru, K., Horikawa, T. (2003). UV-induced skin damage. *Toxicology.* 189: 21–39.

Itahana, K., Mao, H., Jin, A., Itahana, Y., Clegg, H.V., Lindstrom, M.S., Bhat, K.P., Godfrey, V.L., Evan, G.I., Zhang, Y. (2007). Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. *Cancer Cell.* 12: 355- 366.

Ito, S. (2003). The IFPCS presidential lecture: a chemist`s view of melanogenesis. *Pigment Cell Res.* 16: 230–236.

Jackson, R. (1976). The lines of Blaschko: A review and reconsideration: Observations of the cause of certain unusual linear conditions of the skin. *Br J Dermatol.* 95:349-60.

Jackson, M. W. and Berberich, S. J. (2000). MdmX protects p53 from Mdm2-mediated degradation. *Mol. Cell. Biol.* 20, 1001-1007.

Jadali, Z., Eslami, M.B., Sanati, M.H., Mansouri, P., Mahmoudi, M., Maghsoudi, N., Esfahanian, F., (2005). Hepatitis C Virus Antibodies and Vitiligo Disease. *Iranian J Public Health.* 34(1): 23-26.

James, W.D., Berger, T.G., Elston, D.M. (2006). Andrews Diseases of the Skin: Clinical Dermatology, 10th edn. *Saunders Elsivier, Philadelphia, USA.* 860–863.

Javelaud, D., Alexaki, V.I., Mauviel, A. (2008). Transforming growth factor-beta in cutaneous .melanoma. *Pigment Cell Melanoma Res.* 21: 123–132.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Thun, M.J. (2009). Cancer statistics. *CA Cancer J Clin.* 59(4):225–249.

Jimbow, K., Chen, H., Park, J.S., Thomas, P.D. (2001). Increased sensitivity of melanocytes to oxidative stress and abnormal expression of tyrosinase-related protein in vitiligo. *Br J Dermatol.* 144: 55–65.

Jin, Y., Birlea, S.A., Fain, P.R., Gowan, K., Riccardi, S. L., Holland, P.J., Mailloux, C. M., Sufit, A.J., Hutton, S.M., Amadi-Myers, A., Bennett, D.C., Wallace, M.R., McCormack, W.T., Kemp, E.H., Gawkrodger, D.J., Weetman, A.P., Picardo, M., Leone, G., Taïeb, A., Jouary, T., Ezzedine, K., van Geel, N., Lambert, J., Overbeck, A., Spritz. R.A. (2010). Variant of *TYR* and autoimmunity susceptibility loci in generalized vitiligo. *New Engl J Med.* 362:1686–1697.

Jin, Y., Dai, M., Lu, S.Z., Xu, Y., Luo, Z., Zhao, Y., Lu H. (2006). 14-3-3 binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation. *EMBO J.* 25:1207–18

Joazeiro, C.A. and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell.* 102: 549- 552.

Joerger, A.C. and Fersht, A.R. (2008). Structural biology of the tumor suppressor p53. *Annu Rev Biochem.* 77:557-82.

Jones, C., Mackay, A., Grigoriadis, A., Cossu, A., Reis-Filho, J.S., Fulford, L., Dexter, T., Davies, S., Bulmer, K., Ford, E., Parry, S., Budroni, M., Palmieri, G., Neville, A.M., O'Hare, M.J., Lakhani, S.R. (2004). Expression profiling of purified normal human luminal and myoepithelial breast cells: identification of novel prognostic markers for breast cancer. *Cancer Res.* 64: 3037–3045.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F., Caput, D. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell.* 90(4): 809-19.

Kaidbey, K.H., Agin, P.P., Sayre, R.M., Kligman, A.M. (1979). Photoprotection by melanin- a comparison of black and Caucasian skin. *J Am Acad Dermatol.* (1): 249-260.

Karagas, M.R., Greenberg, E.R., Spencer, S.K., Stukel, T.A., Mott, L.A. (1999). Increase in incidence rates of basal cell and squamous cell skin cancer in New Hampshire, USA. New Hampshire Skin Cancer Study Group. *Int J Cancer.* 81(4):555–559.

Karen, H. Vousden, Xin, L.U. (2002). Live or let die: the cell's response to p53. *Nature Reviews Cancer* .2, 594-604.

Kato, Y., Frankenne, F., Noël, A., Sakai, N., Nagashima, Y., Koshika, S., Miyazaki, K., Foidart, J.M. (2000). High production of SPARC/osteonectin/BM-40 in mouse metastatic B16 melanoma cell lines. *Pathol Oncol Res.* *6*: *24*–6.

Kato, Y., Lewalle, J.M., Baba, Y., Tsukuda, M., Sakai, N., Baba, M., Kobayashi, K., Koshika, S., Nagashima, Y., Frankenne, F., Noël, A., Foidart, J.M., Hata, R.I. (2001) Induction of SPARC by VEGF in human vascular endothelial cells. *Biochem Biophys Res Commun.* 287:422– 426.

Kaufman, S. (1957). The enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem.* 226(1): 511-24.

Kaufman, S. (1958). A new cofactor required for the enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem.* 230(2): 931-9.

Kaufman, S. (1959). Studies on the mechanism of the enzymatic conversion of phenylalanine to tyrosine. *J Biol Chem*. 234: 2677-82.

Kaufman, S. (1997). Tetrahydrobiopterin: Basic Biochemistry and Role in Human Disease. *Baltimore, USA: John Hopkins University Press.*

Kauser, S., Thody, A.J., Schallreuter, K.U., Gummer, C.L., Tobin, D.J. (2004). beta-Endorphin as a regulator of human hair follicle melanocyte biology. *J Invest Dermatol.* 123(1): 184-95.

Kawai, H., Lopez-Pajares, V., Kim, M.M., Wiederschain, D., Yuan, Z.M. (2007). RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer Res.* 67: 6026-6030.

Kellogg, E.W., and Fridovich, I. (1975). Superoxide, hydrogen peroxide, and singlet oxygen in lipid peroxidation by a xanthine oxidase system. *J Biol Chem.* 250(22): 8812-7.

Khaitan, B.K., Dash, S., Ramam, M. (2007). A study to characterize segmental vitiligo (conference abstract). *Pigment Cell Res.* 20(3): 249.

Kim, D.S., Park, S.H., Park, K.C. (2003). Transforming growth factor-beta1 decreases melanin synthesis via delayed extracellular signal-regulated kinase activation. *Int J Biochem Cell Biol.* 36(8):1482-91.

Kim, Y.K., Bae, G.U., Kang, J.K., Park, J.W., Lee, E.K., Lee, H.Y., Choi, W.S., Lee, H. W., Han, J.W. (2006). Cooperation of H2O2-mediated ERK activation with Smad pathway in TGF-beta1 induction of p21WAF1/Cip1. *Cell Signal.* (2):236-43.

Ko, L.J. and Prives, C. (1996). p53: puzzle and paradigm. *Genes Dev.* 10(9): 1054-72.

Koga, M. and Tango T. (1988). Clinical features and course of type A and type B vitiligo. *Br J Dermatol.* 118(2):223-8.

Korytowski, W., Pilas, B., Sarna, T., Kalyanaraman, B. (1987). Photoinduced generation of hydrogen peroxide and hydroxyl radicals in melanins. *Photochem Photobiol*. 45:185–190.

Kouzarides,T.(2000). Acetylation: a regulatory modification to rival phosphorylation? *Embo J.* 19: 1176-1179.

Kovacs, S.O. (1998). Vitiligo. *J Am Acad Dermatol.* 38:647-66.

Krasagakis, K., Kruger-Krasagakes, S., Fimmel, S., Eberle, J., Tholke, D., Von Der Ohe, M., Mansmann, U., Orfanos, C. E.(1999). Desensitization of melanoma cells to autocrine TGF-beta isoforms. *J. Cell. Physiol.* 178: 179–187.

Kretsinger RH. (1996). EF-hands reach out. *Nat Struct Biol.* 3(1):12-5.

Krüger, C., and Schallreuter, K.U. (2012). A review of the worldwide prevalence of vitiligo in children/adolescents and adults. *Int J Dermatol.* 51(10):1206-12.

Kubbutat, M.H., Jones, S.N., Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. *Nature.* 387(6630):299-303.

Kudo, H., Hirayoshi, K., Kitagawa, Y., Imamura, S., Nagata, K. (1994). Two collagen-binding proteins, osteonectin and HSP47, are coordinately induced in transformed keratinocytes by heat and other stresses. *Exp Cell Res.* 212(2):219-24.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., Kastan, M.B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc Natl Acad Sci USA.* 89(16): 7491-5.

Kunisada, T., Yamazaki, H., Hayashi, S.I. (2001). Review: ligands for receptor tyrosine kinases expressed in the skin as environmental factors for melanocyte development. *J Investig Dermatol Symp Proc.* 6 (1):6–9.

Kupprion, C., Motamed, K., Sage, E.H. (1998). SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. *J Biol Chem.* 273:29635–29640.

Lane, T.F. and Sage, E.H. (1994). The biology of SPARC, a protein that modulates cellmatrix interactions. *FASEB J.* 8:163–173.

Lane, D.P. (1992). Cancer. p53, guardian of the genome. *Nature.* 358(6381): 15-6.

Lankerani, L., and Baron, E.D. (2004). Photosensitivity to exogenous agents. *J Cutan Med Surg.* 8(6):424–431.

Laptenko, O., and Prives, C. (2006). Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ.*13:951–961.

Lasfar, A. and Cohen-Solal, K.A. (2010). Resistance to transforming growth factor β-mediated tumor suppression in melanoma: are multiple mechanisms in place? *Carcinogenesis.* (10):1710-7.

Lauer, G., Sollberg, S., Cole, M., Flamme, I., Stürzebecher, J., Mann, K., Krieg, T., Eming, S.A. (2000). Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. *J Invest Dermatol.* 115: 12–18.

Laurie, N. A., Donovan, S. L., Zhang, J., Shih, C.-S., Fuller, C. E., Teunisse, A., Johnson, D. A., Wilson, M. W., Rodriguez-Galindo, C., Quarto, M. Francoz, S., Mendrysa, S.M., Guy, R.K., Marine, J.C., Jochemsen, A.G., Dyer, M.A. (2006). Inactivation of the p53 pathway in retinoblastoma. Nature. 444: 61-66.

Lavker, R.M., and Sun, T.T. (1982). Heterogeneity in epidermal basal keratinocytes: Morphological and functional correlations. *Science.* 215: 1239-1240.

Le Poole, I.C., van den Wijngaard, R.M., Westerhof, W., Dutrieux, R.P., Das, P.K. (1993). Presence or absence of melanocytes in vitiligo lesions: an immunohistochemical investigation. *J Invest Dermatol.* 100(6): 816-22.

Le Poole, I.C., and Luiten, R.M. (2008). Autoimmune etiology of generalized vitiligo. *Curr Dir Autoimmun.* 10: 227-43.

Lebwohl, M.G., Heymann, W.R., Berth-Jones, J., Coulson, I. (2006). Treatment of Skin Disease: Comprehensive Therapeutic Strategies, 2nd edn. *Mosby Elsevier, Philadelphia, USA.* 683–687.

Ledda, F., Bravo, A.I., Adris, S., Bover, L., Mordoh, J., Podhajcer, O.L. (1997).The expression of the secreted protein acidic and rich in cysteine (SPARC) is associated with the neoplastic progression of human melanoma. *J Invest Dermatol.* 108*:* 210-4.

Lee, S., Elenbaas, B., Levine, A., Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell.* 81(7): 1013-20.

Leffell, D.J., and Brash, D.E. (1997). Sunlight and the skin cancer. Int J Cancer. 72:231-5.

Leonard, S.S., Harris, G.K., Shi. X.L. (2004). Metal-induced oxidative stress and signal transduction. *Free Rad. Biol. Med.* 37: 1921–1942.

Lerner, A.B. (1959). Vitiligo. *J Invest Dermatol.* 32: 285-310.

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Melino, G., Wang, J.Y. (1999). Structure, function and regulation of p63 and p73. *Cell Death & Differentiation.* 6(12): 1146-53.

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Wang, J.Y., Melino, G. (2000). The p53/p63/p73 family of transcription factors: overlapping and distinct functions. *Journal of Cell Science.* 113(Pt 10): 1661-70.

Levy, C., Khaled, M. and Fisher, D.E. (2006). MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol. Med.* 12: 406-414.

Li, C.Y., Suardet, L., Little, J.B. (1995), Potential role of WAF1/Cip1/p21 as a mediator of TGF-beta cytoinhibitory effect. *J. Biol. Chem.*270(10):4971-4.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science.* 302: 1972-1975.

Li, R. and Botchan M.R. (1993). The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein A and stimulate in vitro BPV-1 DNA replication. *Cell.* 73(6): 1207-21.

Li, W., Sanki, A., Karim, R.Z., Thompson, J.F., Soon Lee, C., Zhuang, L., McCarthy, S. W., Scolyer, R.A. (2006). The role of cell cycle regulatory proteins in the pathogenesis of melanoma. *Pathology.* 38:287-301.

Lien, H.C., Hsiao, Y.H., Lin, Y.S., Yao, Y.T., Juan, H.F., Kuo, W.H., Hung, M.C., Chang, K.J., Hsieh, F.J. (2007). Molecular signatures of metaplastic carcinoma of the breast by large-scale transcriptional profiling: identification of genes potentially related to epithelial-mesenchymal transition. *Oncogene.* 26, 7859–7871.

Lin, A.W. and Lowe, S.W. (2001). Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. *Proc Natl Acad Sci USA.* 98(9): 5025-30.

Lin, J.Y., and Fisher, D.E. (2007). Melanocyte biology and skin pigmentation. *Nature.* 445:843–50.

Linares, L.K., Hengstermann, A., Ciechanover, A., Muller, S., Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *ProcNatlAcadSci USA.* 100: 12009-12014.

Linke, K., Mace, P.D., Smith, C.A., Vaux, D.L., Silke, J., Day, C.L. (2008). Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell Death Differ.* 15: 841- 848.

Liochev, S.I., Fridovich, I. (2002). The Haber-Weiss cycle — 70 years later: an alternative view. *Redox report.* 7: 55–57.

Liu, J.B., Li, M., Yang, S., Gui, J.P., Wang, H.Y., Du, W.H., Zhao, X.Y., Ren, Y.Q., Zhu, Y.G., Zhang, X.J. (2005). Clinical profiles of vitiligo in China: an analysis of 3742 patients. *Clin Exp Dermatol.* 30(4): 327-31.

Lookingbill D. P., Lookingbill g. l., Leppard B. (1995). Actinic damage and skin cancer in albinos in northern Tanzania: findings in 164 patients enrolled in an outreach skin care program. *J Am Acad Dermatol.* (32): 653-658.

Lozano, G. (2007). The oncogenic roles of p53 mutants in mouse models. Curr. Opin. Genet. Dev. 17: 66–70.

Lotti, T., Buggiani, G., Troiano, M., Assad, G.B., Delescluse, J., De Giorgi, V., Hercogova, J. (2008a). Targeted and combination treatments for vitiligo. Comparative evaluation of different current modalities in 458 subjects. *Dermatol Ther.* 21 Suppl 1:S20-6.

Lotti T., Alessia, G., Zanieri, F., Colucci R,Moretti, S. (2008b). Vitiligo: new and emerging treatments. *Dermatol Ther.* 21: 110–117.

Lu, H. and Levine, A.J. (1995). Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc Natl Acad Sci USA* .92(11): 5154-8.

Lutz, M., and Knaus, P. (2002). Integration of the TGF-beta pathway into the cellular signalling network. *Cell Signal*. *14*: 977.

Maker, H.S., Weiss, C., Silides, D.J., Cohen, G. (1981). Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. *J Neurochem.* 36(2): 589-93.

Mancini, F., Gentiletti, F., D'Angelo, M., Giglio, S., Nanni, S., D'Angelo, C., Farsetti, A., Citro, G., Sacchi, A., Pontecorvi, A., Moretti, F. (2004). MDM4 (MDMX) overexpression enhances stabilization of stress-induced p53 and promotes apoptosis. *J Biol Chem.* 279(9):8169-80.

Mancini, F., Di Conza, G., Monti, O., Macchiarulo, A., Pellicciari, R., Pontecorvi, A., Moretti, F. (2010). Puzzling over MDM4-p53 network. *Int J Biochem Cell Biol.* 42(7):1080-3.

Mannick, J.B. and Schonhoff, C.M. (2004). NO means no and yes: regulation of cell signaling by protein nitrosylation. *Free Radic Res.* 38(1): 1-7.

Maresca, V., Flori, E., Briganti, S., Camera, E., Cario-André, M., Taïeb, A., Picardo, M. (2006). UVA-induced modification of catalase charge properties in the epidermis is correlated with the skin phototype. *J Invest Dermatol.* 126(1): 182-90.

Maresca, V., Flori, E., Briganti, S., Mastrofrancesco, A., Fabbri, C., Mileo, A.M., Paggi, M.G., Picardo, M. (2007). Correlation between melanogenic and catalase activity in in vitro human melanocytes: a synergic strategy against oxidative stress. *Pigment Cell Melanoma Res.* 21(2):200-5.

Marine, J.C., Dyer, M.A., Jochemsen, A.G. (2007). MDMX: from bench to bedside. *J Cell Sci.* 120(Pt 3):371-8.

Markovic, S.N., Erickson, L.A., Rao, R.D., Weenig, R.H., Pockaj, B.A., Bardia, A., Vachon, C.M., Schild, S.E., McWilliams, R.R., Hand, J.L., Laman, S.D., Kottschade, L. A., Maples, W.J., Pittelkow, M.R., Pulido, J.S., Cameron, J.D., Creagan, E.T. (2007). Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. *Mayo Clin Proc.* 82(3):364 –380.

Marks, R. (1995). An overview of skin cancers: incidence and causation. *Cancer.* (75): 607-612.

Marles, L.K., Peters, E.M., Tobin, D.J., Hibberts, N.A., Schallreuter, K.U. (2003). Tyrosine hydroxylase isoenzyme I is present in human melanosomes: a possible novel function in pigmentation. *Exp Dermatol.* 12: 61–70.

Marneros, A.G. (2009). Tumor angiogenesis in melanoma. *Hematol Oncol Clin North Am.* 23:431-446, vii-viii.

Marrot, L., Belaidi, J.P., Meunier, J.R., Perez, P., Agapakis-Causse, C. (1999). The human melanocyte as a particular target for UVA radiation and an endpoint for photoprotection assessment. *Photochem Photobiol.* 69:686–693.

Martínez-Esparza, M., Ferrer, C., Castells, M.T., García-Borrón, J.C., Zuasti, A. (2001). Transforming growth factor beta1 mediates hypopigmentation of B16 mouse melanoma cells by inhibition of melanin formation and melanosome maturation. *Int J Biochem Cell Biol.* 33 (10):971-83.

Martinez-Esparza, M., Jimenez-Cervantes, C., Beermann, F., Aparicio, P., Lozano, J. A., Garcia-Borron, J.C. (1997). Transforming growth factor-beta1 inhibits basal melanogenesis in B16/F10 mouse melanoma cells by increasing the rate of degradation of tyrosinase and tyrosinase-related protein-1. *Journal of Biological Chemistry.* 272: 3967–3972.

Martinoli, C., Zocchi, E., Querzola, F., Damiani, G., Zaccheo, D. (1984). Progesterone enhances reactive oxygen intermediates production by cultured human monocytes. *Boll Soc Ital Biol Sper.* 60(10): 1871-7.

Massagué, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* 67: 753-91.

Massi, D., Franchi, A., Borgognoni, L., Reali, U.M., Santucci, M. (1999). Osteonectin expression correlates with clinical outcome in thin cutaneous malignant melanomas. *Hum. Pathol.* 30: 339–344.

Maurer, P., Hohenadl, C., Hohenester, E., Gohring, W., Timpl, R., Engel, J. (1995). The C-terminal portion of BM-40 (SPARC/osteonectin) is an autonomously folding and crystallisable domain that binds calcium and collagen IV. *J. Mol. Biol.* 253: 347–357.

Maurer, P., Mayer, U., Bruch, M., Jenö, P., Mann, K., Landwehr, R., Engel, J., Timpl, R. (1992). High-affinity and low-affinity calcium binding and stability of the multidomain extracellular 40-kDa basement membrane glycoprotein (BM-40/SPARC/osteonectin). *Eur. J. Biochem*. 205: 233–240.

Mehta, H.R., Shah, K.C., Theodore, C. (1973). Epidemiological study of vitiligo in Surat area South Gujarat. *Indian J Med Res.* 61: 145–154.

Migliore, L. and Coppede, F. (2002). Genetic and environmental factors in cancer and neurodegenerative diseases. *Mutat Res.* 512(2-3):135–153.

Mildner, M., Weninger, W., Trautinger, F., Ban, J., Tschachler, E. (1999). UVA and UVB radiation differentially regulate vascular endothelial growth factor expression in keratinocyte derived cell lines and in human keratinocytes. *Photochem Photobiol.* 70: 674–679.

Miller, A.J. and Mihm, M.C. (2006). Melanoma. *N. Engl. J. Med.* 355: 51–65.

Moellmann, G., Klein-Angerer, S., Scollay, D.A., Nordlund, J.J., and Lerner, A.B. (1982). Extracellular granular material and degeneration of keratinocytes in the normally pigmented epidermis of patients with vitiligo. *J Invest Dermatol.* 79(5): 321-30.

Montano, X., Shamsher, M., Whitehead, P., Dawson, K., Newton, J. (1994). Analysis of p53 in human cutaneous melanoma cell lines. *Oncogene.* 9(5):1455-9.

Moretti, S., Spallanzani, A., Amato, L., Hautmann, G., Gallerani, I., Fabiani, M., Fabbri, P. (2002). New insights into the pathogenesis of vitiligo: imbalance of epidermal cytokines at sites of lesions. *Pigment Cell Res*. 15(2): 87-92.

Moretti, S., Amato, L., Bellandi, S., Fabbri, P. (2006). Focus on vitiligo: a generalized skin disorder. *Eur J Inflamm.* 4: 21–30.

Morrone, A., Picardo, M., de Luca, C., Terminali, O., Passi, S., Ippolito, F. (1992). Catecholamines and vitiligo. *Pigment Cell Res.* 5: 65–69.

Mosher, D.B., Fitzpatrick, T.B., Hori, Y., Ortonne, J.P. (1993). Disorders of pigmentation. In: Fitzpatrick T. B. et al., eds. Dermatology in General Medicine, 4th eddition. *New York: McGraw-Hill.* 910-930.

Muthusamy, V., Hobbs, C., Nogueira, C., Cordon-Cardo, C., McKee, P.H., Chin, L., Bosenberg, M.W. (2006). Amplification of CDK4 and MDM2 in malignant melanoma. *Genes Chromosom. Cancer.* 45: 447–454.

Nakamura, S., Kamihagi, K., Satakeda, H., Katayama, M., Pan, H., Okamoto, H., Noshiro, M., Takahashi, K., Yoshihara, Y., Shimmei, M., Okada, Y., Kato, Y. (1996). Enhancement of SPARC osteonectin. synthesis in arthritic cartilage. Increased levels in synovial fluids from patients with rheumatoid arthritis and regulation by growth factors and cytokines in chondrocyte cultures. *Arthritis Rheum.* 39: 539-551.

Naruse, K., Ueda, M., Nagano, T., Suzuki, T., Harada, S., Imaizumi, K., Watanabe, S., Ichihashi, M. (1997). Prevalence of actinic keratosis in Japan. *J Dermatol Sci.* (15): 183-187.

Naski, N., Gajjar, M., Bourougaa, K., Malbert-Colas, L., Fåhraeus, R., Candeias, M.M. (2009). The p53 mRNA-Mdm2 interaction. *Cell cycle.* 8(1):31-4.

Naughton, G.K., Eisinger, M., Bystryn, J.C. (1983). Antibodies to normal human melanocytes in vitiligo. *J Exp Med.* 158(1): 246-51.

Naveh, H.P., Rao, U.N., Butterfield, L.H.(2013). Melanoma-associated leukoderma-immunology in black and white? *Pigment Cell Melanoma Res.* 26(6):796-804.

Nikolova, P.V., Wong, K.B., DeDecker, B., Henckel, J., Fersht, A.R. (2000). Mechanism of rescue of common p53 cancer mutations by second-site suppressor mutations, *The EMBO Journal.* 19: 370 – 378.

Nordlund, J. J. and Lerner, A. B. (1982).Vitiligo. It is important. *Arch Dermatol.* 118: 5-8.

Nordlund, J. J. and Ortonne, J. P. (1992).Vitiligo and depigmentation. *Curr Prob Dermatol.* 4: 3-30.

Nordlund, J.J., Kirkwood, J.M., Forget, B.M., Milton, G., Albert, D.M., Lerner, A.B. (1983). Vitiligo in patients with metastatic melanoma: a good prognostic sign. *J Am Acad Dermatol.*9 (5):689-96.

Nordlund, J.J., Le Poole, I.C., Boissy, R.E. (2008). Vitiligo vulgaris. In Gaspari A. A., Tyring S. K, editors. Clinical and Basic Immunodermatology. Part 4. *Springer London.* pp 661-689.

Nozaki, M., Sakurai, E., Raisler, B.J., Baffi, J.Z., Witta, J., Ogura, Y., Brekken, R.A., Sage, E.H., Ambati, B.K., Ambati, J. (2006). Loss of SPARC-mediated VEGFR-1 suppression after injury reveals a novel antiangiogenic activity of VEGF-A. *J Clin Invest.* 116(2):422-9.

Oettle, A. G. (1963). Skin cancer in Africa. *Natl Cancer Inst Monoger.* (10): 197-214.

Okazaki, K., Uzuka, M., Morikawa, F., Toda, K., Seiji, M. (1976). Transfer mechanism of melanosomes in epidermal cell culture. *J Invest Dermatol.* 67: 541–547.

Oliner, J.D., Pietenpol, J.A., Thiagalingam, S., Gyuris, J., Kinzler, K.W., Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature.* 362: 857-860.

Olivares, C., García-Borrón, J.C., Solano, F. (2002). Identification of active site residues involved in metal cofactor binding and stereospecific substrate recognition in Mammalian tyrosinase. Implications to the catalytic cycle. *Biochemistry*. 41(2): 679-86.

Olson, D.C., Marechal, V. et al. (1993). Identification and characterization of multiple mdm-2 proteins and mdm- 2-p53 protein complexes. *Oncogene*. 8(9): 2353-60.

Olson, J.S., Ballow, D.P., Palmer, G., Massey, V. (1974). The reaction of xanthine oxidase with molecular oxygen. *J Biol Chem.* 249(14): 4350-62.

Ongenae, K., Van Geel, N., Naeyaert, J.M. (2003). Evidence for an autoimmune pathogenesis of vitiligo. *Pigment Cell Res.* 16:90–100.

Orecchia, G. (2000). Neural pathogenesis. In: Hann, S. K., Nordlund J. J. Vitiligo. *Oxford: Blackwell Science.* pp. 142–150.

Ortonne, J.P., Gauthier, Y., Guillet, G., Gauthier, O. *(*1978). Depigmentation cutanee associee au melanome malin. *Ann Dermatol Venereol.* 105: 1043–1052.

Ortonne, J.P. (2008).Vitiligo and other disorders of hypopigmentation. In: Bolognia, J., Jorizzo, J., Rapini, R., editors. Dermatology. Philadelphia: *Mosby Elsevier.* p. 928.

Ortonne, J.P. and Bose, S.K. (1993). Vitiligo: where do we stand? *Pigment Cell Res.* 6(2): 61-72.

Ortonne, J.P., Bahdoran, P., Fitzpatrick, T.B., Mosher, D.B., Hori, Y. (2003). Hypomelanoses and hypermelanoses. In: Fitzpatrick, T. B., Freedberg, I. M., Eisen, A.Z., Wolff, K., Austen, K.F., Goldsmith, L.A., eds. Fitzpatrick's Dermatology in general medicine. 6th ed, *New York: Mc Graw-Hill Proff esional.* p. 839-47.

Osada, T., Chong, G., Tansik, R., Hong, T., Spector, N., Kumar, R., Hurwitz, H.I., Dev, I., Nixon, A.B., Lyerly, H.K., Clay, T., Morse, M.A. (2008). The effect of anti-VEGF therapy on immature myeloid cell and dendritic cells in cancer patients. *Cancer Immunol Immunother.*57:1115-1124.

Oury, T.D., Tatro, L., Ghio, A.J., Piantadosi, C.A. (1995). Nitration of tyrosine by hydrogen peroxide and nitrite. *Free Radic Res.* 23: 537– 547.

Pan, Y. and Chen, J. (2003). MDM2 promotes ubiquitination and degradation of MDMX. *Mol Cell Biol.* 23: 5113- 5121.

Papp, T., Jafari, M., Schiffmann, D. (1996). Lack of p53 mutations and loss of heterozygosity in non-cultured human melanocytic lesions. *J Cancer Res Clin Oncol.* 122: 541–548.

Parks, D.A. and Granger, D.N. (1986). Xanthine oxidase: biochemistry, distribution and physiology. *Acta Physiol Scand Suppl.* 548: 87-99.

Parsad, D., Pandhi, R., Juneja, A. (2003). Effective of oral Ginkgo biloba in treating limited, slowly spreading vitiligo. *Clin Exp Dermatol.* 28: 285–287.

Parsad, D., Pandhi R., Dogra S., Kumar B. (2004). Clinical study of repigmentation patterns with different treatment modalities and their correlation with speed and stability of repigmentation in 352 vitiliginous patches. *J Am Acad Dermatol.* 50(1): 63-7.

Passi, S., Grandinetti, M., Maggio, F., Stancato, A., De Luca, C. (1998). Epidermal oxidative stress in vitiligo. *Pigment Cell Res.* 11(2):81-5.

Pavletich, N.P., Chambers, K.A., Pabo, C.O. (1993). The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. *Genes Dev.* 7(12B): 2556-64.

Pavlović, M.D. (2000). Poremećaji pigmentacije. U: Karadaglić Đ. *Dermatologija. Beograd: Vojnoizdavački zavod.* p. 871-902.

Pawelek, J.M. (1990). Is human melanogenesis stimulated by cyclic AMP? *J Invest Dermatol.* 94: 499–500.

Pekarkova, I., Parara, S., Holecek, V., Stopka, P., Trefil, L., Racek, J., Rokyta, R. (2001). Does exogenous melatonin influence the free radicals metabolism and pain sensation in rat? *Physiol. Res.* 50: 595–602.

Pelengaris, S., and Khan, M. (2006). The molecular biology of cancer. *Blackwell Publishing Ltd.*

Pereg, Y., Lam, S., Teunisse, A., Biton, S., Meulmeester, E., Mittelman, L., Buscemi, G., Okamoto, K., Taya, Y., Shiloh, Y., Jochemsen, A.G. (2006a). Differential roles of ATM and Chk2-mediated phosphorylations of HDMX in response to DNA damage. *Mol Cell Biol.* 26:6819–31.

Pereg, Y., Shkedy, D., de Graaf, P., Meulmeester, E., Edelson-Averbukh, M., Salek, M., Biton, S., Teunisse, A.F., Lehmann, W.D., Jochemsen, A.G., Shiloh, Y. (2005). Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA-damage. *PNAS.* 102: 5056-5061.

Perry, M.E., Mendrysa, S.M., Saucedo, L.J., Tannous, P., and Holubar, M. (2000). p76(MDM2) inhibits the ability of p90(MDM2) to destabilize p53. *J. Biol. Chem.* 275: 5733–5738.

Perry, M.E., Piette, J., Zawadzki, J.A., Harvey, D., Levine, A.J. (1993). The mdm-2 gene is induced in response to UV light in a p53-dependent manner. *ProcNatlAcadSci USA.* 90: 11623-11627.

Peters, E.M., Tobin, D.J., Seidah, N.G., Schallreuter, K.U. (2000). Pro-opiomelanocortin-related peptides, prohormone convertases 1 and 2 and the regulatory peptide 7B2 are present in melanosomes of human melanocytes. *J Invest Dermatol.* 114:430–437.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S. V., Hainaut, P., Olivier, M. (2007b). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: Lessons from recent developments in the IARC TP53 database. *Hum Mutat.* 28: 622–629.

Pey, A.L., Martinez, A., Charubala, R., Maitland, D.J., Teigen, K., Calvo, A., Pfleiderer, W., Wood, J.M., Schallreuter, K.U. (2006). Specific interaction of the diastereomers 7(R) and 7(S) tetrahyydrobiopterin with phenylalanine hydroxylase: implications for understanding primapterinuria and vitiligo. *FASEB J.* (12): 2130-2.

Picardo, M., Grammatico, P., Roccella, F., Roccella, M., Grandinetti, M., Porto G. D., Passi S. (1996). Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma. *J. Invest. Dermatol.* 107: 322- 326.

Porter, P.L., Sage, E.H., Lane, T.F., Funk, S.E., Gown, A.M. (1995). Distribution of SPARC in normal and neoplastic human tissue. *J. Histochem. Cytochem.* 43, 791-800.

Preston, D.S. and Stern, R.S. (1992). Nonmelanoma cancers of the skin. *N Engl J Med*. 327: 1649- 1662.

Prives, C. (1994). How loops, beta sheets, and alpha helices help us to understand p53. *Cell.* 78(4): 543-6.

Prota, G. (1992). Melanins and Melanogenesis. *New York: Academic.*

Prota, G. (1995).The chemistry of melanins and melanogenesis. *Fortsch Chem Organ Natur.* 64: 93-148.

Prota, G., D`Ischia, M., Napolitano, A. (1998). The chemistry of melanins and related metabolites. In: Nordlund, J. J., Boissy, R. E., Hearing, V. J., King, R. A., Ortonne, J-P, editors. The pigmentary system. *New York: Oxford University Press, Inc.* p. 307–32.

Punshi, S.K. (1975). Linear Vitiligo. *Ind J Dermatol Venereol Leprol.* 41(6): 240.

Puolakkainen, P.A., Brekken, R.A., Muneer, S., Sage, E.H. (2004). Enhanced growth of pancreatic tumors in SPARC-null mice is associated with decreased deposition of extracellular matrix and reduced tumor cell apoptosis. *Mol. Cancer Res.* 2: 215–224.

Quan, C., Ren, Y.Q., Xiang, L.H. *et al*. (2010). Genome-wide association study for vitiligo identifies susceptibility loci at 6q27 and the MHC. *Nat Genet.* 42:614-618.

Quaglino, P., Marenco, F., Osella-Abate, S., Cappello, N., Ortoncelli, M., Salomone, B., Fierro, M.T., Savoia, P., Bernengo, M.G. (2010).Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study. *Ann Oncol.* 21(2):409-14

Ragnarsson-Olding, B.K., Karsberg, S., Platz, A., Ringborg, U.K. (2002) Mutations in the TP53 gene in human malignant melanomas derived from sun-exposed skin and unexposed mucosal membranes. *Melanoma Res*. 12: 453–463.

Ram, M. and Shoenfeld, Y. (2007). Harnessing autoimmunity (vitiligo) to treat melanoma: a myth or reality? *Ann N Y Acad Sci.* 1110:410-25.

Raposo, G. and Marks, M.S. (2007). Melanosomes-dark organelles enlighten endosomal membrane transport. *Nat. Rev. Mol. Cell. Biol.* 8: 786-797.

Rass, K., Gutwein, P., Welter, C., Meineke, V., Tilgen, W., Reichrath, J. (2001). DNA mismatch repair enzyme hMSH2 in malignant melanoma: increased immunoreactivity as compared to acquired melanocytic nevi and strong mRNA expression in melanoma cell lines. *Histochem. J.* 33: 459–467.

Raycroft, L., Wu, H.Y., Lozano, G. (1990). Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. *Science.* 249(4972): 1049-51.

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M.E., Tegtmeyer, P. (1995). The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. *Proc Natl Acad Sci USA.* 92(21): 9455-9.

Reed, K.B., M.D., Jerry, D., Brewer, M.D., Christine, M., Lohse, M.S., Kariline, E., Bringe, B.S., Crystal, N., Pruitt, B.S., Lawrence, E., Gibson, M.D. (2012). Increasing Incidence of Melanoma Among Young Adults: An Epidemiological Study in Olmsted County, Minnesota. *Mayo Clin Proc.* 87(4):328-334.

Rempel, S.A., Golembieski, W.A., Ge, S., Lemke, N., Elisevich, K., Mikkelsen, T., Gutierrez, J.A. (1998). SPARC: a signal of astrocytic neoplastic transformation and reactive response in human primary and xenograft gliomas. *J. Neuropathol. Exp. Neurol.* 57: 1112-1121.

Ribeiro N., Sousa, S.R., Brekken, R.A., Monteiro, F.J. (2014). Role of SPARC in Bone Remodeling and Cancer-Related Bone Metastasis. *Journal of Cellular Biochemistry*. 115: 17-26.

Rigel, D.S. (2008). Cutaneous ultraviolet exposure and its relationship to the development of skin cancer. *J Am Acad Dermatol.* 58(5 suppl 2):S129 – S132

Riley, T., Sontag, E., Chen, P., Levine, A. (2008). Transcriptional control of human p53- regulated genes. *Nat Rev Mol Cell Biol.* 9(5): 402-12.

Robert, G., Gaggioli, C., Bailet, O., Chavey, C., Abbe, P., Aberdam, E., Sabatié, E., Cano, A., Garcia de Herreros, A., Ballotti, R., Tartare-Deckert, S. (2006). SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. *Cancer Res.* 66(15):7516-23.

Roberts, A. B. (1998), Molecular and cell biology of TGF-beta. *Miner Electrolyte Metab.* (2-3):111-9.

Rodeck, U., Nishiyama, T., Mauviel, A. (1999). Independent regulation of growth and SMAD-mediated transcription by trans- forming growth factor beta in human melanoma cells. *Cancer Res.* 59: 547–550.

Rodeck, U., Bossler, A., Graeven, U., Fox, F.E., Nowell, P.C., Knabbe, C., Kari, C. (1994). Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. *Cancer Res.* 54: 575–581.

Rokos, H., W. D. Beazley, Schallreuter, K. U. (2002). Oxidative stress in vitiligo: photooxidation of pterins produces H_2O_2 and pterin-6-carboxylic acid. *Biochem Biophys Res Commun.* 292(4): 805-11.

Rokos, H., Moore, J., Hasse, S., Gillbro, J.M., Wood, J. M., Schallreuter, K.U. (2004). *In vivo* Fluorescence Excitation Spectroscopy and *in vivo* FT-Raman Spectroscopy in human skin: Evidence of H_2O_2 oxidation of epidermal albumin in patients with vitiligo. *J Raman Spectrosc.* 35: 125-130.

Rosenberg, S.A. and White, D.E. (1996).Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. *J Immunother Emphasis Tumor Immunol.* 19(1):81-4.

Rossi, F., Della Bianca, V., de Togni, P. (1985). Mechanisms and functions of the oxygen radicals producing respiration of phagocytes. *Comp Immunol Microbiol Infect Dis.* 8(2): 187-204.

Rozan, L.M., and El-Deiry, W.S. (2007). p53 downstream target genes and tumor suppression: a classical view in evolution. *Cell Death Differ.* 14:3–9.

Ruiz-Ramos, R., Lopez-Carrillo, L., Rios-Perez, A.D., De Vizcaya-Ruíz, A., Cebrian, M.E. (2009). Sodium arsenite induces ROS generation, DNA oxidative damage, HO-1 and c-Myc proteins, NF-kappaB activation and cell proliferation in human breast cancer MCF-7 cells. *Mutat Res*. 674(1-2):109-15.

Sagebiel, R.W. and Odland, G.F. (1972). Ultrastructural identification of melanocytes in early human embryos. In: Pigmentation: Its Genesis and Biologic Control. Riley, Y. V. (editor). *Appleton-Century-Crofts, New York.* pp. 43–50.

Sakamuro, D., Sabbatini, P., White, E., Prendergast,G.C. (1997). The polyproline region of p53 is required to activate apoptosis but not growth arrest. *Oncogene.* 15(8): 887-98.

Salasche, S.J. (2000). Epidemiology of actinic keratoses and squamous cell carcinoma. *J Am Acad Dermatol.* (42): S4-S7.

Salem, M.M.A.E.L., Shalbaf, M., Gibbons, N.C.J., Chavan, B., Thornton, J.M., Schallreuter, K.U. (2009). Enhanced DNA binding capacity on up-regulated epidermal wild-type p53 in vitiligo by H2O2-mediated oxidation: a possible repair mechanism for DNA damage. *FASEB Journal*. 3790-3807.

Sander, C.S., Chang, H., Hamm, F., Elsner, P., Thiele, J.J. (2004). Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. *Int J Dermatol*. *43*: 326–35.

Sander, C.S., Hamm, F., Elsner, P., Thiele J.J. (2003). Oxidative stress in malignant melanoma and non-melanoma skin cancer. *Brit. J. Dermatol.* 148 (5): 913-922.

Sangiovanni, S. (1819). Descrizione d'un particolare sisteina di organo cromoforo espansivo dermoideo e dei fenomeni ch'esse produce scopesto nei molluschi cefaloso. *Giornale enciclopedico di Napoli.* 9: 1-15.

Sarna, T., Menon, I.A., Sealy, R.C. (1985). Photosensitization of melanins: a comparative study. *Photochem Photobiol.* 42(5):529-32.

Sasaki, T., Hohenester, E., Göhring, W., Timpl, R. (1998). Crystal structure and mapping by site-directed mutagenesis of the collagen-binding epitope of an activated form of BM-40/SPARC/osteonectin. *EMBO J.* 17:1625-1634.

Sasaki, T., Göhring, W., Mann, K., Maurer, P., Hohenester, E., Knäuper, V., Murphy, G., Timpl, R. (1997). Limited cleavage of extracellular matrix protein BM-40 by matrix metalloproteinases increases its affinity for collagens. *J. Biol. Chem.* 272: 9237–9243.

Saucedo, L.J., Myers, C.D., Perry, M.E. (1999). Multiple murine double minute gene 2 (MDM2) proteins are induced by ultraviolet light.*J. Biol. Chem.* 274: 8161–8168.

Schallreuter, K.U. (1999a). A review of recent advances on the regulation of pigmentation in the human epidermis. *Cell Mol Biol (Noisy-le-grand).* 45(7): 943-49.

Schallreuter, K.U. (1999d). Successful treatment of oxidative stress in vitiligo. *Skin Pharmacol Appl Skin Physiol.* 12(3): 132-8.

Schallreuter, K.U. (2004b). Basic Research: An Update. Vitiligo: Problems and Solutions. T. Lotti and Hercogová. *New York, Basel, Marcel Dekker, Inc.* 29: 65-78.

Schallreuter, K.U. (2005a). Vitiligo. In: Hertl M, ed. Autoimmune Diseases of the Skin Pathogenesis, Diagnosis, Management. *Wien: Springer.* 367–384.

Schallreuter, K.U. (2005b). Oxidative stress in the human epidermis. *G Ital Dermatol Venereol.* 140: 505–514.

Schallreuter, K.U. (2009). Viewpoint 1. *Experimental Dermatology.* 17: 141–144.

Schallreuter, K.U. (2014). Reactive Oxygen Species and Reactive Nitrogen Species in Vitiligo. In: Laher I. ed. Systems Biology of Free Radicals and Antioxidants. *Springer-Verlag Berlin Heidelberg:* pp 3697-3736.

Schallreuter, K.U. and Elwary, S. (2007a). Hydrogen peroxide regulates the cholinergic signal in a concentration dependent manner. *Life Sci.* 80(24-25): 2221-6.

Schallreuter, K.U. and Wood, J.M. (2001). Thioredoxin reductase - its role in epidermal redox status. *J Photochem Photobiol B.* 64(2-3): 179-84.

Schallreuter, K.U., and Pittelkow M.R. (1988). Defective calcium uptake in keratinocyte cell cultures from vitiliginous skin. *Arch Dermatol Res.* 280(3): 137-9.

Schallreuter, K.U., and Salem, M. M. (2010). Vitiligo. What is new? *Hautarzt.* 61(7):578-85.

Schallreuter, K.U., and Wood, J.M. (1999c). The importance of L-phenylalanine transport and its autocrine turnover to L-tyrosine for melanogenesis in human epidermal melanocytes. *Biochem Biophys Res Commun.* 262(2): 423-428.

Schallreuter, K.U., Bahadoran, P., Picardo, M., Slominski, A., Elassiuty, Y.E., Kemp, E.H., Giachino, C., Liu, J.B., Luiten, R.M., Lambe, T., Le Poole, I.C., Dammak, I., Onay, H., Zmijewski, M.A., Dell'Anna, M.L., Zeegers, M P., Cornall, R.J., Paus, R., Ortonne, J.P., Westerhof, W. (2008a). Vitiligo pathogenesis: autoimmune disease, genetic defect, excessive reactive oxygen species, calcium imbalance, or what else? *Exp Dermatol.* 17(2):139-40; discussion 141-60.

Schallreuter, K.U., Behrens-Williams, S., Khaliq, T.P., Picksley, S.M., Peters, E. M., Marles, L.K., Westerhof, W., Miehe, B., Fanghänel,J. (2003). Increased epidermal functioning wild-type p53 expression in vitiligo. *Exp. Dermatol.* 12: 268-277.

Schallreuter, K.U., Büttner, G., Pittelkow, M.R., Wood, J.M., Swanson, N.N., Körner, C. (1994d). Cytotoxicity of 6-biopterin to human melanocytes. *Biochem Biophys Res Commun.* 204(1):43-8.

Schallreuter, K.U., Chavan, B., Rokos, H., Hibberts, N., Panske, A., Wood, J.M. (2005c). Decreased phenylalanine uptake and turnover in patients with vitiligo. *Mol Genet Metab.* 86 Suppl 1: S27-33.

Schallreuter, K.U., Chiuchiarelli, G., Cemeli, E., Elwary, S. M., Gillbro, J.M., Spencer, H. Rokos, J.D., Panske, A., Chavan, B., Wood, J.M., Anderson, D. (2006). Estrogens can contribute to hydrogen peroxide generation and quinone-mediated DNA damage in peripheral blood lymphocytes from patients with vitiligo. *J Invest Dermatol.* 126(5): 1036-42.

Schallreuter, K.U., Elwary, S.M., Gibbons, N.C., Rokos, H. and Wood, J.M. (2004a). Activation/deactivation of acetylcholinesterase by H_2O_2: more evidence for oxidative stress in vitiligo. *Biochem. Biophys. Res. Commun.* 315: 502–508.

Schallreuter, K.U., Gibbons, N.C., Elwary, S.M., Parkin, S.M. and Wood, J.M. (2007b). Calcium-activated butyrylcholinesterase in human skin protects acetylcholinesterase against suicide inhibition by neurotoxic organophosphates. *Biochem. Biophys. Res. Commun.* 355: 1069–1074

Schallreuter, K.U., Gibbons, N.C., Zothner, C., Abou Elloof M.M., Wood J.M. (2007c). Hydrogen peroxide-mediated oxidative stress disrupts calcium binding on calmodulin: more evidence for oxidative stress in vitiligo. *Biochem Biophys Res Commun.* 360(1): 70-5.

Schallreuter, K.U., Kothari, S., Chavan, B., Spencer, J.D. (2007d). Regulation of melanogenesis - controversies and new concepts. *Exp Dermatol.* 17: 395–404.

Schallreuter, K.U., Krüger, C., Rokos, H., Hasse, S., Zothner, C., Panske, A. (2007e). Basic research confirms coexistence of acquired Blaschkolinear Vitiligo and acrofacial Vitiligo. *Arch Dermatol Res.* 299(5-6): 225-30.

Schallreuter, K.U., Krüger, C., Würfel, B.A., Panske, A., Wood, J.M. (2008b). From basic research to the bedside: efficacy of topical treatment with pseudocatalase PC-KUS in 71 children with vitiligo. *Int J Dermatol.* 47(7): 743-53.

Schallreuter, K.U., Lemke, R., Brandt, O., Schwartz, R., Westhofen, M., Montz, R., Berger, J. (1994c). Vitiligo and other diseases: coexistence or true association? Hamburg study on 321 patients. Dermatology. 188(4): 269-75.

Schallreuter, K.U., Levenig, C., Berger, J. (1991a). Vitiligo and cutaneous melanoma. *Dermatologica.* 183: 239- 245.

Schallreuter, K.U., Moore, J., Wood, J.M., Beazley, W.D., Gaze, D.C., Tobin, D.J., Marshall, H.S., Panske, A., Panzig, E., Hibberts, N.A. (1999b). *In vivo* and *in vitro* evidence for hydrogen peroxide (H_2O_2) accumulation in the epidermis of patients with vitiligo and its successful removal by a UVB-activated pseudocatalase. *J Investig Dermatol Symp Proc.* 4(1): 91-6.

Schallreuter, K.U., Rübsam, K., Gibbons, N.C., Maitland, D.J., Chavan, B., Zothner, C., Rokos, H., Wood, J.M. (2007f). Methionine Sulfoxide Reductases A and B Are Deactivated by Hydrogen Peroxide (H_2O_2) in the Epidermis of Patients with Vitiligo. *J Invest Dermatol.* 128(4):808-15.

Schallreuter, K.U., Salem, M.A., Gibbons N.C., Maitland, D.J., Marsch E., Elwary, S.M., Healey, A.R. (2012b). Blunted epidermal L-tryptophan metabolism in vitiligo affects immune response and ROS scavenging by Fenton chemistry, part 2: Epidermal H_2O_2/ONOO(-)-mediated stress in vitiligo hampers indoleamine 2,3-dioxygenase and aryl hydrocarbon receptor-mediated immune response signaling. *FASEB J.* (6):2471-85.

Schallreuter, K.U., Salem, M.A., Gibbons, N.C., Martinez, A., Slominski, R., Lüdemann, J., Rokos, H. (2012a). Blunted epidermal L-tryptophan metabolism in vitiligo affects immune response and ROS scavenging by Fenton chemistry, part 1: Epidermal H_2O_2/ONOO(-)-mediated stress abrogates tryptophan hydroxylase and dopa decarboxylase activities, leading to low serotonin and melatonin levels. *FASEB J.* (6):2457-70.

Schallreuter, K.U., Salem, M.A., Holtz, S., Panske, A. (2013). Basic evidence for epidermal H_2O_2/ONOO (-)-mediated oxidation/nitration in segmental vitiligo is supported by repigmentation of skin and eyelashes after reduction of epidermal H_2O_2 with topical NB-UVB-activated pseudocatalase PC-KUS. *FASEB J.* 27(8):3113-22.

Schallreuter, K.U., Tobin, D.J., Panske, A. (2002). Decreased photodamage and low incidence of non-melanoma skin cancer in 136 sun-exposed Caucasian patients with vitiligo. *Dermatology.* 204(3): 194-201.

Schallreuter, K.U., Wazir, U., Kothari, S., Gibbons, N.C., Moore, J., Wood, J.M. (2004c). Human phenylalanine hydroxylase is activated by H_2O_2: a novel mechanism for increasing the L-tyrosine supply for melanogenesis in melanocytes. *Biochem Biophys Res Commun.* 322: 88–92.

Schallreuter, K.U., Wood, J. M., Pittelkow, M., R., Büttner, G., Swanson, N.N., Körner, C., Ehrke, C. (1994d). Increased monoamine oxidase A activity in the epidermis of patients with vitiligo. *Arch Dermatol Res.* 288(1): 14-8.

Schallreuter, K.U., Wood, J.M., Berger, J. (1991b). Low catalase levels in the epidermis of patients with vitiligo. *J Invest Dermatol.* 97(6): 1081-5.

Schallreuter, K.U., Wood, J.M., Lemke, K.R., Levenig, C. (1995a). Treatment of vitiligo with a topical application of pseudocatalase and calcium in combination with short-term UVB exposure: a case study on 33 patients. *Dermatology.* 190(3): 223-9.

Schallreuter, K.U., Wood, J.M., Pittelkow, M. R., Gütlich, M., Lemke, K. R., Rödl, W., Swanson, N. N., Hitzemann, K., Ziegler, I. (1994a). Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterin. *Science.* 263(5152): 1444-6.

Schallreuter, K.U., Wood, J.M., Pittelkow, M.R., Swanson, N.N., Steinkraus, V. (1993). Increased in vitro expression of beta 2-adrenoceptors in differentiating lesional keratinocytes of vitiligo patients. *Arch Dermatol Res.* 285(4):216-20.

Schallreuter, K.U., Wood, J.M., Ziegler, I., Lemke, K.R., Pittelkow, M.R., Lindsey, N.J., Gütlich, M. (1994b). Defective tetrahydrobiopterin and catecholamine biosynthesis in the depigmentation disorder vitiligo. *Biochim Biophys Acta.* 1226(2): 181-92.

Schallreuter-Wood, K. U., Pittelkow, M.,R., Swanson, N. N. (1996). Defective calcium transport in vitiliginous melanocytes. *Arch Dermatol Res.* 288: 11–13.

Schweikardt T, Olivares C, Solano F, Jaenicke E, Garcia-Borron J C, Decker H. (2007). A three-dimensional model of mammalian tyrosinase active site accounting for loss of function mutations. *Pigment Cell Res.* 20: 394–401.

Scott, G., Leopardi, S., Printup, S., Madden, B. C. (2002). Filopodia are conduits for melanosome transfer to keratinocytes. *J Cell Sci.* 115: 1441–1451.

Seabra, M. C. and Coudrier, E. (2004). Rab GTPases and myosin motors in organelle motility. *Traffic.* 5: 393- 399.

Sehgal, V.N., and Srivastava, G. (2007).Vitiligo: compendium of clinico-epidemiological features. *Indian J Dermatol Venereol Leprol.* 73: 149–156.

Seidah, N.G., Chretien, M., Day, R. (1994). The family of subtilisin/kexin like pro-protein and pro-hormone convertases: divergent or shared functions. *Biochimie.* 76:197-209.

Sengupta, S., and Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. *Nat. Rev. Mol. Cell Biol.* 6, 44–55.

Setty, S. R., Tenza, D., Truschel, S. T., Chou, E., Sviderskaya, E. V., Theos, A. C., Lamoreux, M. L., Di Pietro, S. M., Starcevic, M., Bennett, D. C. et al., (2007). BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol. Biol. Cell.* 18: 768-780.

Sevilla, L.M., Nachat, R., Groot, K.R., Klement, J.F., Uitto, J, Djian, P., Määttä, A., Watt, F.M. (2007). Mice deficient in involucrin, envoplakin, and periplakin have a defective epidermal barrier. *J Cell Biol.* 179(7): 1599-612.

Shajil, E.M., Chatterjee, S., Agrawal, D., Bagchi, T., Begum, R. (2006).Vitiligo: pathomechanisms and genetic polymorphism of susceptible genes. *Indian J Exp Biol.* 44:526-39.

Shalbaf, M., Gibbons, N.C., Wood, J.M., Maitland, D.J., Rokos, H., Elwary, S.M., Marles, L.K., Schallreuter K.U. (2008). Presence of epidermal allantoin further supports oxidative stress in vitiligo. *Exp Dermatol.* 17(9): 761-70.

Sharp, D.A., Kratowicz, S.A., Sank, M.J. George, D.L. (1999). Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J Biol Chem.* 274: 38189-38196.

Sharquie, K.E., Mehenna, S.H., Naji, A.A., Al-Azzawi, H. (2004). Inflammatory changes in vitiligo: stage I and II depigmentation. *Am J Dermatopathol.* 26(2): 108-12.

Shaulsky, G., Goldfinger, N., Tosky, M.S., Levine, A.J., Rotter, V. (1991). Nuclear localization is essential for the activity of p53 protein. *Oncogene.* 6(11): 2055-65.

Shibanuma, M., Kuroki, T. et al. (1991). Release of H_2O_2 and phosphorylation of 30 kilodalton proteins as early responses of cell cycle-dependent inhibition of DNA synthesis by transforming growth factor beta 1. *Cell Growth Differ.* 2(11): 583-91.

Shieh, S.Y., Ikeda, M., Taya, Y., Prives, C. (1997). DNA-damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell. 91*: 325-334.

Shier, D., Butler, J., Lewis, R. (1999). In Hole's Human Anatomy and Physiology. 8th Eddition. *McGraw Hill.* 160–183.

Shvarts, A., Steegenga, W.T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R.C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A.J., Jochemsen, A.G. (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J.* 15:5349–57.

Sies, H., and Cadenas, E. (1985). Oxidative stress: damage to intact cells and organs. *Philos Trans R Soc Lond B Biol Sci.* 311(1152): 617-31.

Singh, M., Singh, G., Kanwar, A.J., Belhaj, M.S. (1985). Clinical pattern of vitiligo in Libya. *Int J Dermatol.* 24(4): 233-5.

Singh, S.K., Kurfurst, R., Nizard, C., Schnebert, S., Perrier, E., Tobin, D.J. (2010). Melanin transfer in human skin cells is mediated by Filopodia a model for homotypic and heterotypic lysosome-related organelle transfer. *FASEB J*. 24: 1-14.

Slominski, A., Paus, R., Bomirski, A. (1989). Hypothesis: possible role for the melatonin receptor in vitiligo: discussion paper. *J R Soc Med.* 82(9): 539-41.

Slominski, A., Wortsman, J., Mazurkiewicz, J. E., Matsuoka, L., Dietrich, J., Lawrence, K., Gorbani, A., Paus, R. (1993). Detection of proopiomelanocortin- derived antigens in normal and pathologic human skin. *J Lab Clin Med.* 122: 658-666.

Slominski, A. Paus, R. Schadendorf, D. (1993). Melanocytes as "sensory" and regulatory cells in the epidermis. *J Theor Biol.* 164 (1): 103–20.

Slominski, A., Tobin, D.J., Shibahara, S., Wortsman, J. (2004). Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev.* 84(4): 1155-228.

Smith C., Marks, A., Lieberman, M. (2004). Oxygen Toxicity and Free Radical Injury" in: Marks' Basic Medical Biochemistry, A clinical approach. *Baltimore, Williams and Wilkins.*

Soengas, M.S., Lowe, S.W. (2003). Apoptosis and melanoma chemoresistance. Oncogene. 22:3138-3151.

Song, H., and Xu, Y. (2007).Gain of function of p53 cancer mutants in disrupting critical DNA damage response pathways. *Cell Cycle.* 6:1570–1573.

Soto, J.L., Cabrera, C.M., Serrano, S., Lopez-Nevot, M.A. (2005) Mutation analysis of genes that control the G1/S cell cycle in melanoma: TP53, CDKN1A, CDKN2A, and CDKN2B. *BMC Cancer.* 5:36.

Soussi, T., and Beroud, C (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer.* 1:233-240.

Sparrow, L. E., Soong, R., Dawkins, H.J., Iacopetta, B.J., Heenan, P.J. (1995). P53 gene mutation and expression in naevi and melanomas. *Melanoma Res.* 5:93-100.

Spencer, J.D., B. Chavan, Marles, L.K., Kauser, S., Rokos, H., Schallreuter, K.U. (2005). A novel mechanism in control of human pigmentation by -melanocyte-stimulating hormone and 7- tetrahydrobiopterin. *J Endocrinol. 187*(2): 293-302.

Spencer, J.D., Gibbons, N.C., Rokos, H., Peters, E.M., Wood, J.M., Schallreuter, K.U. (2007). Oxidative stress via hydrogen peroxide affects

proopiomelanocortin peptides directly in the epidermis of patients with vitiligo. *J Invest Dermatol.* 127:411–20.

Spencer, J.D., and Schallreuter, K.U. (2009). Regulation of pigmentation in human epidermal melanocytes by functional high-affinity beta-melanocyte-stimulating hormone/melanocortin-4 receptor signaling. *Endocrinology.* 150(3):1250-8.

Srivastava, A., Ralhan, R., Kaur, J. (2003). Angiogenesis in cutaneous melanoma: pathogenesis and clinical implications. *Microsc Res Tech.* 60(2):208-224.

Stad, R., Ramos, Y.F.M., Little, N., Grivell, S., Attema, J., van der Eb, A.J., Jochemsen, A.G. (2000). Hdmx stabilizes Mdm2 and p53. *J. Biol. Chem.* 275: 28039-28044.

Stadtman, E.R. (2004).Cyclic oxidation and reduction of methionine residues of proteins in antioxidant defense and cellular regulation. *Arch Biochem Biophys.* 1: 423(1):2-5.

Sterner, D.E., and Berger, S.L. (2000). Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev.* 64: 435-459.

Stohs S.J. and Bagchi D. (1995). Oxidative mechanisms in the toxicity of metal-ions. *Free Rad. Biol. Med.* 18: 321–336.

Straume, O., Akslen, L.A. (2001). Expression of vascular endothelial growth factor, its receptors (FLT-1, KDR) and TSP-1 related to microvessel density and patient outcome in vertical growth phase melanomas. *Am J Pathol.* 159: 223–235.

Sturm, R.A., Satyamoorthy, K., Meier, F., Gardiner, B.B., Smit, D.J., Vaidya, B., and Herlyn, M. (2002). Osteonectin/SPARC induction by ectopic beta (3) integrin in human radial growth phase primary melanoma cells. *Cancer Res.* 62: 226–232.

Suárez, B., López-Abente, G., Martínez, C., Navarro, C., Tormo, M. J., Rosso, S., Schraub, S., Gafà, L., Sancho-Garnier, H., Wechsler, J., Zanetti, R. (2007). Occupation and skin cancer: the results of the HELIOS-I multicenter case-control study. *BMC Public Health.* 7: 180.

Sun, X., Xu, A., Wei, X., Ouyang, J., Lu, L., Chen, M., Zhang, D. (2006).Genetic epidemiology of vitiligo: a study of 815 probands and their families from south China. *Int J Dermatol.* 45:1176-81.

Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., Finke, l. T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science.* 13; 270(5234):296-9.

Swift, J.A. (1964). Transfer of melanin granules from melanocytes to the cortical cells of human hair. *Nature.* 203: 976–977.

Szczurko, O., Boon, H.S. (2008).A systematic review of natural heath product treatment for vitiligo. *BMC Dermatol.* 8: 2.

Tai, I.T., Tang, M.J. (2008). SPARC in cancer biology: Its role in cancer progression and potential for therapy. *Drug Resistance Updates.* 11: 231-246.

Taïeb, A., and Picardo, M. (2007). The definition and assessment of vitiligo: a consensus report of the Vitiligo European Task Force. *Pigment Cell Res.* 20(1):27-35.

Tang, Y., Zhao, W., Chen, Y., Zhao, Y., Gu, W. (2008). Acetylation is indispensable for p53 activation. *Cell.* 133:612–26.

Termine, J.D., Kleinman, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., Martin, G.R. (1981). Osteonectin, a bone-specific protein linking mineral to collagen. *Cell.* 26, 99–105.

Teulings, H.E., Overkamp, M., Ceylan, E., Nieuweboer-Krobotova, L., Bos, J.D., Nijsten, T., Wolkerstorfer, A.W., Luiten, R.M., van der Veen, J.P. (2013). Decreased risk of melanoma and nonmelanoma skin cancer in patients with vitiligo: a survey among 1307 patients and their partners. *Br J Dermatol.* 168(1):162-71.

Teulings, H.E., Willemsen, K.J., Glykofridis, I., Krebbers, G., Komen, L., Kroon, M.W., Kemp, E.H., Wolkerstorfer, A., van der Veen, J.P., Luiten, R.M., Tjin, E.P. (2015). The antibody response against MART-1 differs in patients with melanoma-associated leucoderma and vitiligo. *Pigment Cell Melanoma Res.* 27(6):1086-96.

Thannickal, V.J., Day, R M., Klinz, S.G., Bastien, M.C., Larios, J.M., Fanburg, B.L. (2000). Ras-dependent and -independent regulation of reactive oxygen species by mitogenic growth factors and TGF-beta1. *FASEB J.* 14: 1741–1748.

Thannickal, V.J., Hassoun, P.M., et al. (1993). Enhanced rate of H_2O_2 release from bovine pulmonary artery endothelial cells induced by TGF-beta 1. *Am J Physiol.* 265(6 Pt 1): L622-6.

Theos, A.C., Tenza, D., Martina, J.A., Hurbain, I., Peden, A.A., Sviderskaya, E.V., Stewart, A., Robinson, M.S., Bennett, D.C., Cutler, D.F. et al., (2005). Functions of adaptor protein (AP)-3 and AP-1 in tyrosinase sorting from endosomes to melanosomes. *Mol. Biol. Cell.* 16: 5356-5372.

Theos, A.C., Truschel, S.T., Tenza, D., Hurbain, I., Harper, D.C., Berson, J.F., Thomas, P.C., Raposo, G., Marks, M.S. (2006). A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. *Dev Cell.* 10(3):343-54.

Thompson, J.F., Scolyer, R.A., Kefford, R.F. (2005). Cutaneous melanoma. *Lancet.* 365:687-701.

Thut, C.J., Chen, J.L., Klemm, R., Tjian, R. (1995). p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. *Science.* Jan 6: 267(5194):100-4.

Tobin, D.J., Swanson, N.N., Pittelkow, M.R., Peters, E.M., Schallreuter, K.U. (2000). Melanocytes are not absent in lesional skin of long duration vitiligo. *J Pathol.* 191(4): 407-16.

Toledo, F., Wahl, G.M. (2007). MDM2 and MDM4: p53 regulators as targets in anticancer therapy. *Int J Biochem Cell Biol.* 39(7-8):1476-82.

Toledo,. F., Wahl, G.M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat Rev Cancer.* 6:909–923.

Tonsi, A. (2004). Vitiligo and its management update: a review. *Pak J Med Sci.* 20: 242–247.

Tsao, H., Atkins, M.B., Sober, A.J. (2004). Management of cutaneous melanoma. *N Engl J Med.* 351:998-1012.

Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M., and Haupt, Y. (1999). Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. *Embo J.* 18:1805-1814.

Van Belle, P., Rodeck, U., Nuamah, I., Halpern, A.C., Elder, D.E. (1996) Melanoma associated expression of transforming growth factor-beta isoforms. *Am. J. Pathol.* 148, 1887–1894.

Valencia, J.C., Rouzaud, F., Julien, S., Chen, K.G., Passeron, T., Yamaguchi, Y., Abu-Asab, M., Tsokos, M., Costin, G.E., Yamaguchi, H., Jenkins, L.M., Nagashima, K., Appella, E., Hearing, V.J. (2007). Sialylated core 1 O-glycans influence the sorting of Pmel17/gp100 and determine its capacity to form fibrils. *J Biol Chem.* 282(15):11266-80.

Van den Bossche, K., Naeyaert, J.M., Lambert, J. (2006). The quest for the mechanism of melanin transfer. *Traffic.* 7: 769-778.

Van der Vliet, A., O'Neill, C.A., Halliwell, B., Cross, C.E., Kaur, H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite. Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett.* 339: 89–92.

Venot, C., Maratrat, M., Dureuil, C., Conseiller, E., Bracco, L., Debussche, L. (1998). The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. *Embo J.* 17(16): 4668-79.

Vile, G.F. (1997). Active oxygen species mediate the solar ultraviolet radiation-dependent increase in the tumour suppressor protein p53 in human skin fibroblasts. *FEBS Lett.* 412: 70-74

Vogelstein, B., Lane, D., Levine, A.J. (2000). Surfing the p53 network. *Nature.* 408(6810): 307-10.

Vousden, K.H. and Lane, D.P. (2007). p53 in health and disease. *Nat Rev Mol Cell Biol.* 8(4): 275-83.

Vousden, K.H., Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer*. 2:594–604.

Wade, M. and Wahl, G.M. (2009). Targeting Mdm2 and Mdmx in cancer therapy: better living through medicinal chemistry? *Mol Cancer Res.* 7:1–11.

Walker, K.K. and Levine A.J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci USA.* 93(26): 15335-40.

Wang, Y., Reed, M., Wang, P., Stenger, J.E., Mayr, G., Anderson, M.E., Schwedes, J.F., Tegtmeyer, P. (1993). p53 domains: identification and characterization of two autonomous DNA- binding regions. *Genes Dev.* 7(12B): 2575-86.

Wasmeier, C., Hume, A.N., Bolasco, G., Seabra, M.C. (2008). Melanosomes at a glance. *J Cell Science.* 121:3995-3999.

Weeraratna, A.T., Jiang, Y., Hostetter, G., Rosenblatt, K., Duray, P., Bittner, M., Trent, J.M. (2002). Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. *Cancer Cell.* 1 (3): 279–88.

Weninger, W., Uthman, A., Pammer, J., Pichler, A., Ballaun, C., Lang, I.M., Plettenberg, A., Bankl, H.C., Stürzl, M., Tschachler, E.(1996). Vascular endothelial growth factor production in normal epidermis and in benign and malignant epithelial skin tumors. *Lab Invest.* 75: 647–657.

Whibley, C., Pharoah, P.D., Hollstein, M. (2009). p53 polymorphisms: cancer implications. *Nat Rev Cancer.* 9(2): 95-107.

Wiseman, H., Kaur, H., Halliwell, B. (1995). DNA damage and cancer: measurement and mechanism. *Cancer Lett.* 93(1):113–120.

Wolff, K., Goldsmith, L.A., Katz, S.I., Gilchrest, B.A., Paller, A.S., Leffell, D.J. (2007). Fitzpatrick`s Dermatology in General Medicine, 7th edn, Vol. I. *Mac Graw Hill, USA.* 616–621.

Wong G.S., Rustgi, A.K. (2013) Matricellular proteins: Priming the tumour microenvironment for cancer development and metastasis. *Br J Cancer.* 108,755–61.

Wong, G.H., and Goeddel, D.V. (1988). Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science.* 242: 941–944.

Wood, J.M., Schallreuter-Wood, K.U., Lindsey, N.J., Callaghan, S., Gardner, L.G. (1995). A specific tetrahydrobiopterin binding domain on tyrosinase controls melanogenesis. *Biochem Biophys Res Commun.* 206: 480–485.

Wood, J.M., Chavan, B., Hafeez, I., Schallreuter, K.U. (2004). Regulation of tyrosinase by tetrahydropteridines and H_2O_2. *Biochem Biophys Res Commun.* 325: 1412–1417.

Wood, J.M., Chavan, B., Hafeez, I., Schallreuter, K.U. (2005). Regulation of tyrosinase by tetrahydropteridines-What is real? A critical reanalysis of H. Wojtasek's view .*Biochem Biophys Res Commun.* 331: 891–893.

Wood, J.M. and Schallreuter K.U. (2006). UVA-irradiated pheomelanin alters the structure of catalase and decreases its activity in human skin. *J Invest Dermatol.* 126(1): 13-4.

Wood, J.M., Gibbons, N.C., Chavan, B., Schallreuter, K.U. (2008). Computer simulation of heterogeneous single nucleotide polymorphisms in the catalase gene indicates structural changes in the enzyme active site, NADPH-binding and tetramerization domains: a genetic predisposition for an altered catalase in patients with vitiligo? *Exp Dermatol.* 17(4): 366-71.

Wood, J.M., Decker, H., Hartmann, H., Chavan, B., Rokos, H., Spencer, J.D., Hasse, S., Thornton, M.J., Shalbaf, M., Paus, R., and Schallreuter, K.U. (2009). "Senile hair graying: H_2O_2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair. *FASEB J.* 23(7):2065-75.

World Health Organization. (1994). Ultraviolet radiation. Environmental health criteria 160. Geneva.

Wu, X., Bowers, B., Rao, K., Wei, Q. and Hammer, J.A. (1998). Visualization of melanosome dynamics within wild-type and dilute melanocytes suggests a paradigm for myosin V function in vivo. *J. Cell Biol.* 143: 1899-1918.

Würfel, F. (1999) Untersuchungen zur Ätiopathogenese der Vitiligo:Können virale Erkrankungen bei der Manifestation dieser Erkrankung möglicherweise eine Rolle spielen? Dissertation Universität Hamburg / Germany

Xie, Z., Chen, D., Jiao, D., Bystryn, J.C. (1999). Vitiligo antibodies are not directed to tyrosinase. *Arch Dermatol.* 135: 417–422.

Yaar, M., and Gilchres,t B.A. (1991). Human melanocyte growth and differentiation: a decade of new data. *J Invest Dermato.* l97 (4): 611-7.

Yamamoto, M., Takahashi, H. (1993). Immunohistochemical detection of the p53 oncoprotein in tumours of melanocytic origin. *Virchows Arch A Pathol Anat Histopathol.* 422(2):127-32.

Yamamoto, O., and Bhawan, J. (1994). Three modes of melanosome transfers in Caucasian facial skin: hypothesis based on an ultrastructural study. *Pigment Cell Res.* 7: 158–169.

Yan, Q., Sage, E.H. (1999). SPARC, a matricellular glycoprotein with important biological functions. In: 50th Annual Meeting of the Histochemical-*Society. Bethesda, MD: Histochemical Soc Inc. Pp*. 1495–1505.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dötsch, V., Andrews, N.C., Caput, D., McKeon, F. (1998). p63, a p53 homolog at 3q27-29,

encodes multiple products with transactivating, death-inducing, and dominantnegative activities. *Mol Cell.* 2(3): 305-16.

Yang, E., Kang, H.J., Koh, K.H., Rhee, H., Kim, N.K., Kim, H. (2007). Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. *Int. J. Cancer.* 121: 567–575.

Yiu, G.K., Chan, W.Y., Ng, S.W., Chan, P.S., Cheung, K.K., Berkowitz, R.S., Mok, S.C. (2001). SPARC (secreted protein acidic and rich in cysteine) induces apoptosis in ovarian cancer cells. *Am. J. Pathol.* 159: 609–622.

Yoshida, H., Kunisada T., Grimm T., Nishimura E.K., Nishioka E., Nishikawa S.I. (2001). Review: melanocyte migration and survival controlled by SCF/c-kit expression. *J Invest Dermatol Symp Proc.* 6(1):1–5.

Yoshida, H., Kunisada T., Kusakabe M., Nishikawa S., Nishikawa S.I. (1996). Distinct stages of melanocyte differentiation revealed by analysis of nonuniform pigmentation patterns. *Development.* 122(4): 1207–1214.

Yu, J., and Zhang, L. (2005). The transcriptional targets of p53 in apoptosis control. *Biochem Biophys Res Commun.* 331:851–858.

Zaidi, M.R., Day, C.P., Merlino, G. (2008). From UVs to metastases: modeling melanoma initiation and progression in the mouse. *J Invest Dermatol.* 128(10):2381-91.

Zerp, S.F., van Elsas, A., Peltenburg, L.T., Schrier, P.I. (1999). p53 mutations in human cutaneous melanoma correlate with sun exposure but are not always involved in melanomagenesis. *Br J Cancer.* 79: 921–926.

Zhang, X.J., Liu, J.B., Gui, J.P., Li, M., Xiong, Q.G., Wu, H.B., Li, J.X., Yang, S., Wang, H.Y., Gao, M., Yang, J., Yang, Q. (2004). Characteristics of genetic epidemiology and genetic models for vitiligo. *J Am Acad Dermatol.* 51(3): 383-90.

Zsebo, K.M., Williams, D.A., Geissler E.N., Broudy, V.C., Martin, F.H., Atkins, H. L., Hsu, R.Y., Birkett, N.C., Okino, K.H., Murdock. D.C., et al. (1990). Stem cell factor is encoded at the SI locus of the mouse and is the ligand for the c-Kit receptor. *Cell.* 63: 213-214.